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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants: Denisa D. Wagner and Robert C. Johnson
Serial No. Not yet assigned
Filed: October 10, 1997
For: METHOD FOR TREATING AND PREVENTING ATHEROSCLEROSIS

BOX FWC
Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

DECLARATION UNDER 37 C.F.R. §1.132

I, Denisa D. Wagner, declare that:

1. I am a Professor in the Department of Pathology at Harvard Medical School, and Senior Investigator at The Center for Blood Research, Boston, Massachusetts. My Curriculum Vitae is attached as Appendix I. I am an inventor of the above-identified application. I am highly skilled in the art regarding the subject matter to which the above-identified application pertains and am aware of the knowledge of the ordinary person skilled in the art.

2. I have examined the Office Action for the parent application Serial No. 08/377,798 dated November 13, 1996, for the above-identified continuation application. I am familiar with the present claims of this application which are directed to methods for treating or inhibiting atherosclerosis in a mammal by administering an agent which inhibits interaction between P-selectin and a ligand P-selectin and between E-selectin and a ligand of E-selectin.

3. I am familiar with the prior art cited by the Examiner: Kogan et al., Rao et al., Seekamp et al., Ross, Rohrer et al. and DeAmbrosi.

4. Based on my background, training and experience in this field, it is my opinion that it is not inherent that every agent which inhibits interaction between P-selectin and a P-selectin ligand also inhibits interaction between E-selectin and an E-selectin ligand. It was known in the art at the time that the parent application was filed, that in fact there are agents which inhibit P-selectin but not E-selectin, and agents which inhibit E-selectin but not P-selectin. See, e.g., attached Exhibit A: Cecconi et al., J. Biol. Chem. 269:15060-15066 (1994)(describes agent which inhibits P-selectin but not E-selectin), and Exhibit B: Lenter et al., J. Cell Biol. 125:471-481 (1994)(describes ligands which bind to P-selectin but not to E-selectin and other ligands which bind to E-selectin but not to P-selectin).

5. Based on my background, training and experience in this field, it is my opinion that it was not obvious at the time that the parent application was filed for one skilled in the art to select agents which inhibit both P-selectin and E-selectin for enhanced effectiveness in treating atherosclerosis. It was not known in the art at that time that both E- and P-selectin are involved in cardiovascular disease and chronic inflammation.

6. Based on my background, training and experience in this field, it is my opinion that an important distinction exists between "acute inflammation" and "chronic inflammation." See, e.g., attached Exhibit C: Robbins, in "Pathologic Basis of Disease," 5th Ed., R.S. Cotran, M.D., V. Kumar, M.D. and S.L. Robbins, M.D., W.B. Saunders Co., Philadelphia, PA, pp. 51-76 (1994). "Acute inflammation" is of relatively short duration and is involved in processes such as wound repair, infection and reperfusion injury. It involves mainly recruitment of neutrophils. "Chronic inflammation," on the other hand, is of longer duration and is associated predominantly with the recruitment of monocytes and T-cells. As is known to one skilled in the art, atherosclerosis is a special example of chronic

inflammation. This specificity of recruitment, combined with smooth muscle cell proliferation and dependence on cholesterol ingestion by the monocytes/macrophages, makes atherosclerosis a unique process. At the time that the parent application was filed, there were no known adhesion receptors that were specific for recruitment of monocytes and T-cells.

7. Based on my background, training and experience in this field, it is my opinion that it was known to one skilled in the art at the time that the parent application was filed, that P-selectin was a receptor that mediated rolling of many types of white blood cells, was rapidly expressed on activated cells, and was stored in preformed granules that could be rapidly released from these cells. Moreover, as was known by one skilled in the art at the time, P-selectin was involved in early recruitment of neutrophils in experimentally-induced inflammation. See attached Exhibit D: Mayadas et al., Cell 74:541-554 (1993)(recruitment of neutrophils was delayed in P-selectin-deficient mice for two hours and then occurred at a rate identical to wild-type mice). Similarly, delay in the recruitment of neutrophils in wound healing has been reported to occur only in the first two hours in P-selectin-deficient mice after injury. See attached Exhibit E: Subramaniam et al., Am. J. Pathology 150:1701-1709 (1997). And, recruitment of macrophages three to seven days post wounding has been reported to be normal in P-selectin-deficient mice, with wound healing occurring at the same rate as in wild-type mice. See attached Exhibit E: Subramaniam et al., Am. J. Pathology 150:1701-1709 (1997). It is my opinion that to a person skilled in the art, these results indicate that P-selectin plays a role in acute inflammation and injury, but not in chronic processes such as atherosclerosis.

8. Based on my background, training and experience in this field, to the best of my knowledge the first published indication that P-selectin plays a role in long-term chronic inflammation

came in 1995, after the filing date of the parent application. See attached Exhibit J: Johnson et al., Blood 86:1106-1114 (1995) which showed reduced macrophage recruitment 48 hours after induction of experimental inflammation. See also attached Exhibit F: Subramaniam et al., J. Exp. Med. 181:2277-2282 (1995). This paper reported that recruitment of inflammatory cells, including $CD4^+$ T cells, in a contact hypersensitivity response, was reduced in P-selectin-deficient mice. This result was a big surprise to those skilled in the art.

9. Based on my background, training and experience in this field, to the best of my knowledge it was not until 1997, after the filing date of the parent application, that the first published report appeared demonstrating a role for any adhesion receptor molecule, and specifically for P-selectin, in atherosclerosis. See attached Exhibit G: Johnson et al., J. Clin. Invest. 99:1037-1043 (1997).

10. Based on my background, training and experience in this field, it is my opinion that a role for P-selectin in chronic inflammation such as atherosclerosis was contrary to the state of knowledge of those skilled in the art at the time that the parent application was filed, and was certainly not "obvious" to those skilled in the art.

11. Based on my background, training and experience in this field, it is my opinion that a role for E-selectin in chronic inflammation such as atherosclerosis was not experimentally supported at the time that the parent application was filed. Indeed, even as of the instant date, no defects in any inflammatory or wound healing models have been reported for E-selectin-deficient mice unless antibodies inhibitory of P-selectin are also used. See attached Exhibit K: Labow et al., Immunity 1:700-720 (1994)(published after the filing date of the parent application).

12. Based on my background, training and experience in this field, to the best of my knowledge, it was not until 1996, after the filing of the parent application, that it was reported, by myself and others, that major defects existed in P- and E-selectin double deficient mice. See attached Exhibit H: Frenette et al., Cell 84:563-574 (1996), and attached Exhibit I: Bullard et al., J. Exp. Med. 183:2329-2336 (1996). These papers showed conclusively for the first time that the two endothelial selectins, P and E together, are crucial for leukocyte recruitment to sites of inflammation. Prior to these papers, these selectins were known to be involved in leukocyte rolling (with minor or no consequences on leukocyte recruitment)(see attached Exhibit D: Mayadas et al., Cell 74:541-554 (1993)), and it is my opinion that it was believed by persons skilled in the art that it was the adhesion molecules responsible for leukocyte firm adhesion to endothelium (belonging to the immunoglobulin and integrin family of receptors), that were crucial for the final transmigration of leukocytes to sites of inflammation in the tissues.

13. Based on my background, training and experience in this field, it is my opinion that a role for E-selectin in atherosclerosis had not been shown at the time that the parent application was filed.

14. Attached hereto as Exhibit L are five figures illustrating the results of experiments performed in my laboratory which support the surprising and unexpected results obtained from mice being deficient in both P-selectin and E-selectin, as opposed to being deficient just for P-selectin, in inhibiting atherosclerotic lesions on arterial walls. Experimental protocols were performed as described in Exhibit G: Johnson et al., J. Clin. Invest. 99:1037-1043 (1997). Fig. 1 illustrates that the size of aortic sinus lesions in LDL-receptor (LDLR)-deficient mice on an atherogenic (high cholesterol and

fat) diet is significantly smaller in P- and E-selectin double deficient mice than in wild-type or just P-selectin-deficient mice. Fig. 2 consists of photographs of entire aortae of LDLR-deficient mice on an atherogenic diet, and illustrates that the percentage area of the aortae that have atherosclerotic lesions is significantly smaller in P- and E-selectin double deficient mice than in wild-type mice. Fig. 3 illustrates that there are significantly smaller aortic sinus lesions in LDLR-deficient mice on an atherogenic diet in P- and E-selectin double deficient mice than in wild-type or just P-selecin-deficient mice. Fig. 4 illustrates that the size of atherosclerotic lesions in the aortic sinus of LDLR-deficient mice, as a function of the length of time on an atherogenic diet, is significantly smaller for up to at least 37 weeks, in P- and E-selectin double deficient mice than in wild- type or just P-selectin-deficient mice. Fig. 5 illustrates that the percentage of mice with calcification in the aortic sinus of LDLR-deficient mice on an atherogenic diet, is significantly less in P- and E-selectin double deficient mice than in wild type mice.

15. Based on my knowledge, training and experience in this field, it is my opinion that no combination of the cited prior art teaches or suggests a method for treating or inhibiting atherosclerosis by providing an agent for inhibiting interaction between P-selectin and a ligand of P-selectin and between E-selectin and a ligand of E-selectin. Nor does any combination of the cited prior art suggest the advantages that are present in applicants' invention.

I further declare that statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or

imprisonment, or both, under §1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the above-identified application or any patent issuing thereon.

Date: 10/8/97

Denisa D. Wagner

DENISA D. WAGNER, Ph.D.

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Appendix I

CURRICULUM VITAE

DENISA D. WAGNER, Ph.D.

ADDRESS: The Center for Blood Research
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Phone: (617) 278-3344
FAX: (617) 278-3368

BORN: Prague, Czechoslovakia - December 30, 1950; U.S. Citizen

EDUCATION: Universite de Geneve, Switzerland - Biochemistry
Diploma of Biochemistry, 1975, with distinction

Massachusetts Institute of Technology, Cambridge, MA
Biology - Ph.D., 1980

FACULTY POSITIONS:

Professor of Pathology, Harvard Medical School, Boston, MA.
1997-present.

Associate Professor of Pathology, Harvard Medical School, Boston, MA.
1994-1997.

Senior Investigator, The Center for Blood Research, Boston, MA.
1994-present.

Associate Professor of Anatomy and Cellular Biology, Tufts University School of Medicine, Boston, MA. 1989-1994.

Associate Professor of Medicine, Tufts University School of Medicine and Member, Special and Scientific Staff, New England Medical Center, Boston, MA. 1987-1994.

Assistant Professor of Biophysics, University of Rochester School of Medicine and Dentistry, Rochester, New York. 1985-1987.

Assistant Professor of Medicine, University of Rochester School of Medicine and Dentistry, Rochester, New York. 1982-1987.

Senior Instructor in Medicine, University of Rochester School of Medicine and Dentistry, Rochester, New York. 1981-1982.

AWARDS: Established Investigator Award, American Heart Association, Biosynthesis of von Willebrand protein by endothelial cells. 1986-1991.

XIth ISTH Congress award in recognition of an outstanding communication, 1987.

MEMBERSHIPS, OFFICES, AND COMMITTEE ASSIGNMENTS IN PROFESSIONAL SOCIETIES:

1980-Present	American Society for Cell Biology
1982-Present	American Society of Hematology
1982-Present	International Society of Thrombosis and Haemostasis
1983-Present	Council on Thrombosis, American Heart Association
1985-Present	International Society of Thrombosis and Haemostasis, subcommittee on von Willebrand factor
1991-1996	American Heart Association, Vascular Wall Biology Study Committee
1992-Present	American Heart Association, Council on Thrombosis Executive Committee
1993-1995	American Heart Association, Council on Thrombosis Long-Range Planning Committee
1994-1996	American Heart Association, Council on Thrombosis Membership Committee (Chairman)
1994-Present	American Association for the Advancement of Science
1994-Present	North American Vascular Biology Organization
1995-1998	American Society of Hematology, Scientific Subcommittee on Thrombosis
1997-Present	North American Vascular Biology Organization, Councilor

EDITORIAL BOARDS:

1993-Present	Molecular Biology of the Cell
1994-Present	Journal of Clinical Investigation

PUBLICATIONS
DENISA D. WAGNER, Ph.D.

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3. Hynes RO, Destree AT, Perkins ME and **Wagner DD.** Cell surface fibronectin and oncogenic transformation. J Supramolecular Str 11:95-104, 1979.

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10. **Wagner DD** and Marder VJ. Biosynthesis of von Willebrand protein by human endothelial cells: identification of a large precursor polypeptide chain. J Biol Chem 258:2065-2067, 1983.

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12. Sporn LA, Rubin P, Marder VJ and **Wagner DD.** Irradiation induces release of von Willebrand protein from endothelial cells in culture. Blood 64:567-570, 1984.

13. **Wagner DD** and Marder VJ. Biosynthesis of von Willebrand protein by human endothelial cells: processing steps and their intracellular localization. J Cell Biol 99:2123-2130, 1984.

14. Sporn LA, Chavin SI, Marder VJ and **Wagner DD.** Biosynthesis of von Willebrand protein by human megakaryocytes. J Clin Invest 76:1102-1106, 1985.

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77. Dong ZM and **Wagner DD**. Leukocytes fighting against obesity. Molecular Psychiatry, In press, 1997.

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EXHIBIT
A

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 269, No. 21, Issue of May 27, pp. 15060–15066, 1994
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Inositol Polyanions

NONCARBOHYDRATE INHIBITORS OF L- AND P-SELECTIN THAT BLOCK INFLAMMATION*

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Selectins are cell adhesion molecules known to support the initial attachment of leukocytes to inflamed vascular endothelium through their recognition of carbohydrate ligands such as the tetrasaccharide sialyl Lewis^x (Neu5Acα2–3Galβ1–4(Fucα1–3)GlcNAc-). In the present study, we describe the inhibition of L- and P-selectin function by inositol polyanions, simple 6-carbon ring structures that have multiple ester-linked phosphate or sulfate groups. In a purified component competition assay, binding of L- and P-selectin-Ig fusion proteins to immobilized bovine serum albumin-sialyl Lewis^x neoglycoprotein was inhibited by inositol hexakisphosphate ($InsP_6$, IC_{50} = 2.1 ± 1.4 μM and 160 ± 40 μM), by inositol pentakisphosphate ($InsP_5$, IC_{50} = 1.4 ± 0.2 and 260 ± 40 μM), and by inositol hexakissulfate ($InsS_6$, IC_{50} = 210 ± 80 μM and 2.8 ± 0.9 mM); E-selectin-Ig binding was unaffected. Inositol polyanions diminished the adhesion of LS180 colon carcinoma cells to plates coated with L- and P-selectin-Ig but not with E-selectin-Ig. Inositol polyanions blocked polymorphonuclear leukocyte (PMN) adhesion to COS cells expressing recombinant transmembrane P-selectin but not to those expressing E-selectin. In addition, inositol polyanions diminished PMN adhesion to activated endothelial cells under rotation-induced shear stress, a process known to require L-selectin function. *In vivo*, the effects of inositol polyanions were studied in two murine models of acute inflammation. Intravenously administered $InsP_6$ (two doses of 40 μmol/kg) inhibited PMN accumulation in thioglycolate-induced inflammation (55 ± 10% inhibition) and in zymosan-induced inflammation (61 ± 4% inhibition). $InsP_5$ and $InsS_6$ also inhibited inflammation in these models, although higher doses were required for $InsS_6$. In conclusion, inositol polyanions are noncarbohydrate small molecules that inhibit L- and P-selectin function *in vitro* and inflammation *in vivo*.

Selectins are transmembrane glycoproteins containing an N-terminal lectin domain, an epidermal growth factor repeat, and a discrete number of complement regulatory-like repeats (reviewed in Ref. 1). E-selectin is synthesized and expressed by cytokine- and endotoxin-activated endothelial cells with peak expression occurring at 4 h. P-selectin is stored in granules of platelets and endothelial cells and can be rapidly redistributed to the cell surface following activation by certain mediators, including thrombin. The third family member, L-selectin, is constitutively expressed by neutrophils, monocytes, and most lymphocytes and participates in the regulation of leukocyte rolling and lymphocyte homing; a substantial portion of cell surface-expressed L-selectin is shed upon cellular activation. E-, P-, and L-selectin bind sLe^x[1] and related oligosaccharides (1); however, recent studies suggest differences in binding specificities and affinities (2–6). Other studies have highlighted the importance of specific proteins in the presentation of carbohydrate ligands (7–13).

Soluble oligosaccharides related to sLe^x and sLe^a (Neu5Acα2–3Galβ1–3(Fucα1–4)GlcNAc-) can inhibit adhesive functions of selectins (6, 14, 15) and have anti-inflammatory activity in rodent models of P- and E-selectin-dependent inflammation (16–18). Three observations prompted us to look for other classes of selectin inhibitors. First, sLe^x and sLe^a appear to interact relatively weakly with L- and P-selectin. For example, sLe^x concentrations in excess of 5 mM are required to block 50% of L-selectin-dependent interactions *in vitro* (2, 19). Second, the complex structures of these oligosaccharides pose significant obstacles for large-scale synthesis. Third, previous studies have shown that a variety of phosphate- or sulfate-containing carbohydrates can interact with L- and P-selectin (6, 20–29). For example, PPME, the polyphosphomonoester core of *Hansenula holstii* *O*-phosphonomannan (30), is a blocker of L-selectin-dependent adhesion (20, 25, 26). High concentrations (5–10 mM) of monophosphated monosaccharides can also block L- and P-selectin-dependent adhesion *in vitro* (20, 23, 26, 27). In addition, two highly sulfated macromolecules, fucoidan, a polysaccharide produced by brown algae, and heparin, a glycosaminoglycan produced by mast cells, interact with both L- and P-selectin *in vitro* (6, 21, 22, 28, 29) and

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[1] The abbreviations used are: sLe^x, sialyl Lewis x; sLe^a, sialyl Lewis a; $InsP_6$, D-*myo*-inositol 1,2,3,4,5,6-hexakisphosphate; $InsS_6$, D-*myo*-inositol 1,2,3,4,5,6-hexakissulfate; $InsP_5$, D-*myo*-inositol 1,3,4,5,6-pentakisphosphate; Ins(1,4,5)P_3, D-*myo*-inositol 1,4,5-trisphosphate; Ins(3,5,6)P_3, D-*myo*-inositol 3,5,6-trisphosphate (equivalent to L-Ins (1,4,5)P_3); GroPIns(4,5)P_2, 1-(α-glycerophospho)-D-*myo*-inositol 4,5-bisphosphate; GroPIns, 1-(α-glycerophospho)-D-*myo*-inositol; $InsP_1$, D-*myo*-inositol 1-monophosphate; Glc(2,3,4,6)P_4, D-glucose 2,3,4,6-tetrakisphosphate; mAb, monoclonal antibody; DPBS, Dulbecco's phosphate-buffered saline (containing Ca^{2+} and Mg^{2+}); BSA, bovine serum albumin; BSA-sLe^x, bovine serum albumin conjugated to sLe^x; PMN, polymorphonuclear leukocytes; ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; HUVEC, human umbilical vein endothelial cells; HSA, human serum albumin; BAL, bronchoalveolar lavage; PBS, phosphate-buffered saline.

inhibit leukocyte rolling (31–33) and inflammation *in vivo* (29). Together, these observations suggested the possibility that multiple phosphate or sulfate groups can contribute to the formation of ligands for at least two of the selectins. In the present study, we attempt to better define selectin interactions with polyphosphated or polysulfated structures. We focused on inositol polyanions, simple 6-carbon ring structures derived from D-*myo*-inositol (1,2,3,5-trans-4,6 cyclohexanehexol) by esterification with phosphate or sulfate groups (Fig. 1). The effects of inositol polyanions on selectin function were examined using a direct binding assay (competition ELISA), as well as several assays measuring selectin-dependent cell adhesion. Finally, inositol polyanions were tested *in vivo* in two mouse models of peritoneal inflammation and in a rat model of lung inflammation.

MATERIALS AND METHODS

Chemicals—$InsP_6$ (dodecasodium salt), $InsS_6$ (hexapotassium salt), Ins(1,4,5)P_3 (potassium salt), inositol 1-monophosphate (cyclohexylammonium salt), *myo*-inositol, 2,3-bisphosphoglycerate (pentasodium salt), pentasodium tripolyphosphate hexahydrate ($Na_5P_3O_{10}$), and trisodium trimetaphosphate ($Na_3P_3O_9$) were from Sigma. $InsP_8$ was also purchased from Calbiochem (La Jolla, CA), as were $InsP_5$ (decasodium salt), GroPIns(4,5)P_2 (trilithium salt), Ins(3,5,6)P_3 (trilithium salt), and GroPIns (lithium salt). D-glucose 2,3,4,6-tetrakisphosphate was synthesized starting from α-D-glucose, which was converted to the benzyl α-D-glucopyranoside. Polyphosphitylation with dibenzyl *N,N*-diethyl phosphorous amidite and tetrazole (34) followed by *in situ* oxidation with peracetic acid gave the fully protected benzyl α-D-glucopyranoside 2,3,4,6-tetrakis (dibenzyl) phosphate in good yield (73%). Benzyl protecting groups were quantitatively removed by catalytic hydrogenation with H_2 on Pd/C (10%) to give pure D-glucose 2,3,4,6-tetrakisphosphate as the free acid. All products gave satisfactory ^1H NMR, ^{31}P NMR, and fast atom bombardment mass spectra.

Stock solutions to be used for animal experiments were freshly prepared in sterile pyrogen-free 0.9% NaCl (saline; Abbott Laboratories, North Chicago, IL), adjusted to pH 7.4 with HCl, and sterilized by 0.22-μm filtration. Endotoxin content was tested using a quantitative chromogenic limulus amebocyte lysate assay (LAL, Whittaker Bioproducts, Inc., Walkersville, MD).

Proteins and Antibodies—Selectin-immunoglobulin fusion proteins (selectin-Ig) are recombinant chimeric molecules containing the lectin domain, epidermal growth factor repeat, and one (L-selectin-Ig), two (P-selectin-Ig), or six (E-selectin-Ig) complement regulatory repeats coupled to the hinge, CH2, and CH3 regions of human IgG1 (3, 6, 35–37). Selectin-Ig cDNAs in pCDM7 and pNUT vectors allowed transient expression in COS-1 cells (38) and stable expression in baby hamster kidney cells (39), respectively. Selectin-Igs were affinity-purified from culture media using protein A-agarose (Pierce Chemical Co.) or protein G-Sepharose (Pharmacia LKB Biotechnology Inc.) (38). BSA-sLe^x was kindly provided by Chembiomed, LTD (Edmonton, Alberta, Canada). Horseradish peroxidase-conjugated goat anti-human IgG (Fc-specific) antibody was from Jackson Immunoresearch Laboratories, Inc. (West Grove, PA). Fluorescein-conjugated anti-human IgG antibody was from Cappel (Durham, NC). BSA (Pentex Fraction V, protease-free) was from Miles, Inc. (Kankakee, IL). Anti-E-selectin mAb H18/7 (protein A-purified antibody) was generated by immunization of mice with human endothelial cells (40). The following murine mAbs were provided as gifts: anti-P-selectin mAbs G1 (protein A-purified antibody, from R. McEver, Oklahoma City, OK) (41) and anti-L-selectin mAb LAM 1.3 (ascites, from T. Tedder, Boston, MA) (42).

Competition ELISA—Competition ELISAs using P- and E-selectin-Ig (10–30 nM) were performed essentially as described (6). Briefly, polystyrene microwell plates (cat. 25801, Corning Glass, Newark, CA) were coated with unconjugated BSA or BSA-sLe^x neoglycoprotein (0.11 μg/well in 75 μl of 50 mM sodium carbonate/bicarbonate buffer, pH 9.5) by incubation overnight at 4 °C. Wells were washed and blocked with 20 mg/ml BSA in assay buffer (20 mM HEPES pH 7.4, 150 mM NaCl, 2 mM $CaCl_2$). P- and E-selectin-Ig and serially diluted test compounds were incubated in BSA-sLe^x-coated wells for 3 h at room temperature. After washing with assay buffer, peroxidase-conjugated goat anti-human IgG antibody (1:3000) was added to the wells and incubated for 30 min. Wells were washed and incubated with a chromogenic substrate for peroxidase (*o*-phenylene-diamine dihydrochloride, 0.8 mg/ml) in 50 mM sodium citrate, 50 mM sodium phosphate buffer (pH 5.0) containing 0.015% (vol/vol) H_2O_2. Bound selectin-Ig was determined by measuring the optical density at 450 nm at intervals of 12–30 s in a V_{max} microplate reader (Molecular Devices, Inc., Menlo Park, CA); an endpoint determination at 490 nm was made after stopping the color development in the linear range by addition of 4 N H_2SO_4. Specific binding to BSA-sLe^x was determined by subtracting the signal generated in wells coated with unconjugated BSA incubated with 20 nM solutions of selectin-Ig (typically less than 10% of the maximal signal). For L-selectin-Ig (2), 20 nM fusion protein was allowed to form multimeric aggregates with peroxidase-conjugated anti-immunoglobulin antibody (1:6,000) for 30 min before incubation with the inhibitors on the BSA-sLe^x-coated plate. Subsequent steps were as described above. IC_{50} values (concentrations of compound that reduced selectin-Ig binding to 50% of the maximal) were calculated by fitting data from a titration curve to the equation: fraction of maximal binding = $IC_{50} \div (IC_{50} + [\text{compound}])$ using nonlinear least squares analysis software (Origin, Microcal Inc., Northampton, MA).

Cells and Culture Conditions—Human LS180 colon carcinoma cells, human HL60 promyelocytic leukemia cells, and SV40-transformed simian kidney fibroblast COS-1 cells were purchased from American Type Culture Collection (Rockville, MD) and maintained in culture as recommended. Baby hamster kidney cells were cultured in Dulbecco's modified Eagle's medium/F-12 (Whittaker) containing 10% fetal bovine serum (FBS; Hyclone, Logan, UT) and subcultured using versene (Life Technologies, Inc.). Primary cultures of human umbilical vein endothelial cells (HUVEC) were obtained from Clonetics Corp. (San Diego, CA). HUVEC were grown in Medium 199 (Life Technologies, Inc.) containing 20% FBS, 50 μg/ml endothelial cell growth supplement, and 100 μg/ml heparin (Sigma) and subcultured (1:3 split ratio) using trypsin/versene (Life Technologies, Inc.). HUVEC were used for adhesion experiments at passage 2–4. PMN were prepared from EDTA-anticoagulated blood by sodium metrizoate-dextran density gradient centrifugation (Polymorphprep, Nycomed Pharma As, Oslo, Norway) and washed twice with DPBS (without Ca^{2+} and Mg^{2+}, Whittaker) at room temperature. Contaminating red blood cells were lysed by a brief exposure (30 s) to hypotonic buffer (ice-cold DPBS diluted 1:10 in sterile distilled water) followed by the addition of 1/10 of a volume of sterile hypertonic DPBS (10 ×, Whittaker). PMN were washed by centrifugation in ice-cold DPBS (without Ca^{2+} and Mg^{2+}) and suspended in ice-cold 1% human serum albumin (HSA; Cat. 521100, Alpha Therapeutic Corporation, Los Angeles, CA) in RPMI 1640 medium (Whittaker).

LS180 Cell Adhesion to Protein A-captured Selectin-Ig—Cell adhesion assays on immobilized selectin-Ig were performed essentially as described (3, 6). Briefly, Nunclon Terasaki microwell plates (Cat. 136528, Nunc, Naperville, IL) were coated overnight at 4 °C with 5 μl/well 50 mM sodium carbonate buffer (pH 9.5) containing recombinant protein A (10 μg/ml, Chemicon, Temecula, CA). Protein A-coated plates were washed with DPBS, incubated for 1 h with L-, P-, or E-selectin-Ig in DPBS (20, 20, and 5 μg/ml, respectively), washed again, and blocked with DPBS containing 1% HSA. LS180 cells were harvested by brief trypsinization, washed twice by centrifugation, and suspended at 1.5 × 10^6 cells/ml in DPBS containing 1% HSA; 5 μl/well of the suspension was added to the wells and incubated for 30 min at 4 °C. After washing to remove unbound cells, adherent cells were fixed with glutaraldehyde (2.5% in DPBS) and counted microscopically. In each experiment, wells coated with CD8-Ig fusion protein were used as control. To measure inhibition of cell adhesion, 5 μl/well of serially diluted solutions of inositol or inositol polyanions in DPBS were added to selectin-Ig-coated plates and incubated for 30 min at 4 °C before the addition of LS180 cell suspension.

PMN Adhesion to COS Cells Transfected with cDNAs Encoding L-, P- and E-selectin—pCDM7/pCDM8 vectors containing cDNAs encoding full-length transmembrane forms of L-, P-, or E-selectin were transfected into COS-1 cells using DEAE-dextran as described (38). After 24 h, transfected cells were harvested by brief trypsin treatment, transferred to coverslips coated with 0.1% gelatin (G8–500, Fisher, Pittsburgh, PA), and cultured in 24-well culture plates for 48–72 h to allow for cell surface expression of selectins. Wells were washed with DPBS and incubated with 0.5 ml of DPBS-1% HSA alone or containing inositol or inositol polyanions for 30 min at 4 °C. PMN suspensions (0.5 ml at 2 × 10^6 cells/ml) were then added and allowed to adhere for 30 min at 4 °C. Nonadherent PMN were removed by immersion of the coverslips in DPBS. Adherent cells were fixed with 2.5% glutaraldehyde in DPBS and counted. PMN adhesion was quantitated as number of rosettes (COS cells with three or more bound PMN) per 100 transfected COS cells. Transfection efficiency (typically 10–25%) was assessed in each experiment by incubating COS cells with mAbs specific for L-, P-, and E-selectin, followed by reaction with a fluorescein-conjugated anti-im-

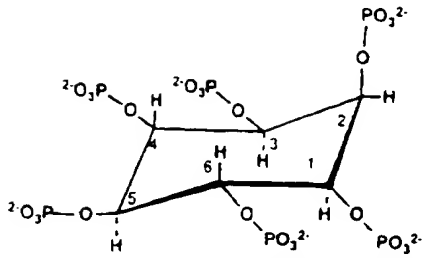

FIG. 1. **Structure of D-*myo*-inositol 1,2,3,4,5,6-hexakisphosphate ($InsP_6$).**

munoglobulin antibody. The same protocol was used for adhesion experiments performed with LS180 and HL60 cell suspensions.

PMN Adhesion to Activated Endothelial Monolayers—HUVEC were grown to confluence within 2.2 cm diameter circles (25) on glass microscope slides coated with 0.1% gelatin. Monolayers were washed with DPBS and incubated for 4–8 h at 37 °C in Medium 199 containing 20% FBS and 200 units/ml human recombinant tumor necrosis factor α (Biogen Research Corp., Cambridge, MA). Monolayers were washed again and incubated for 30 min at 4 °C in Medium 199 containing 5% FBS. After removal of medium, PMN suspension (0.5 ml, 2×10^6/ml) was added and allowed to adhere for 30 min at 4 °C. The adhesion assays were performed both in static and in nonstatic conditions (on a rotating platform, 64 rpm). After washing to remove unbound PMN, glass slides were fixed in DPBS containing 2.5% glutaraldehyde, and adherent PMN were counted microscopically. To determine adhesion-blocking activity, PMN were incubated in RPMI 1640 medium with 5% FBS alone or containing inositol, $InsP_6$, or anti-L-selectin monoclonal antibody LAM 1.3 (25), for 30 min at 4 °C before their addition to activated endothelial monolayers. In some experiments, endothelial monolayers were incubated with anti-E-selectin monoclonal antibody H18/7 (40) for 30 min at 4 °C before the addition of PMN.

Peritoneal Inflammation—Male 4–5-week-old BALB/c mice (20–24 g) were injected intraperitoneally with 1 ml of 3% thioglycollate broth (lot 622462, Clinical Standard Laboratories, Inc., Rancho Dominguez, CA) or with 1 ml of saline containing 0.5 mg of zymosan (Sigma). Control animals were injected with saline. After 40 and 80 min, animals received slow intravenous injections of saline or saline containing $InsP_6$, $InsS_5$, or inositol (0.2 ml/injection). $InsP_6$ was injected at 5–40 μmol/kg/injection (total of 10–80 μmol/kg) and $InsS_6$ and inositol at 40 or 200 μmol/kg/injection (total of 80 or 400 μmol/kg). Mice were sacrificed 120 min after the intraperitoneal injection. Cells within the peritoneal cavity were collected by lavage with 10 ml of ice-cold DPBS containing 10 units/ml of heparin and counted in a hemocytometer. The percentage of PMN was assessed using cytospin preparations (Shandon Inc., Pittsburgh, PA) stained with Wright-Giemsa stain (Diff-Quik, Baxter, McGaw Park, IL). In separate experiments, $InsP_5$ (2–160 μmol/kg) or inositol were given as single 0.2-ml subcutaneous injections 3 min after intraperitoneal injection of thioglycollate. In some experiments, total white blood cell counts were obtained using a Sysmex F-800 hematology analyzer (Baxter), and the percentage of PMN was assessed using smear preparations stained with Diff-Quik. No toxic effects of $InsP_6$ and $InsS_6$ were observed by using the injection protocols described above. Toxicity due to rapid intravenous injection of high dose $InsP_6$ (~600 μmol/kg) has been previously reported (43).

Lung Inflammation—Lung inflammation was induced in rats (male, 200–250 g) by intratracheal injection of endotoxin (5 μg/rat of *Salmonella typhosa* lipopolysaccharide, Sigma, lot 126F4020, in 0.5 ml of saline) as described (44). After 2 h, a first intravenous injection (0.5 ml) of $InsP_6$ (20 μmol/kg) or saline was given; a second injection was given 4 h after the intratracheal injection. Rats were sacrificed 6 h after the intratracheal injection. Bronchoalveolar lavage (BAL) was performed by washing the lungs with 7 ml of PBS injected through a tracheal cannula and repeated 6–7 times/rat. Cells within the BAL fluid were counted with an hematology analyzer, and the percentage of PMN was assessed using cytospin preparations stained with Diff-Quik. Samples of venous blood withdrawn from the tail were used to determine peripheral blood PMN content.

RESULTS

Inositol Polyanions Block the Binding of L- and P-selectin-Ig to BSA-sLe^x in a Competition ELISA—Inositol hexakisphosphate ($InsP_6$, Fig. 1) and inositol hexakissulfate ($InsS_6$), but not inositol, inhibited binding of L- and P-selectin-Ig to immobilized BSA-sLe^x neoglycoprotein (Fig. 2, *A* and *B*). The IC_{50} of $InsP_6$ on L-selectin-Ig binding was 2.1 ± 1.5 μM; $InsS_6$ was less active, with an IC_{50} value of 210 ± 80 μM. $InsP_6$ and $InsS_6$ also blocked P-selectin-Ig binding to immobilized BSA-sLe^x (IC_{50} = 160 ± 40 μM and 2.8 ± 0.9 mM, respectively), whereas they failed to inhibit the binding of E-selectin-Ig at concentrations up to 5 mM (Fig. 2*C*). $InsP_5$ also inhibited L- and P-selectin-Ig binding with IC_{50} values of 1.4 ± 0.2 and 260 ± 40 μM, respectively. As shown in Table I, inositol trisphosphates ($Ins(1,4,5)P_3$, $Ins(3,5,6)P_3$, and $GroPIns(4,5)P_3$) also inhibited L-selectin binding, while $Glc(2,3,4,6)P_4$ was inactive up to 2 mM. Inositol-based structures with low phosphate content (inositol, $InsP_1$, and GroPIns) and polyphosphates (sodium trimetaphosphate, sodium tripolyphosphate, and 2,3-bisphosphoglycerate) did not inhibit selectin binding to BSA-sLe^x up to 2 mM. None of the test compounds inhibited E-selectin-Ig binding to BSA-sLe^x up to 2 mM.

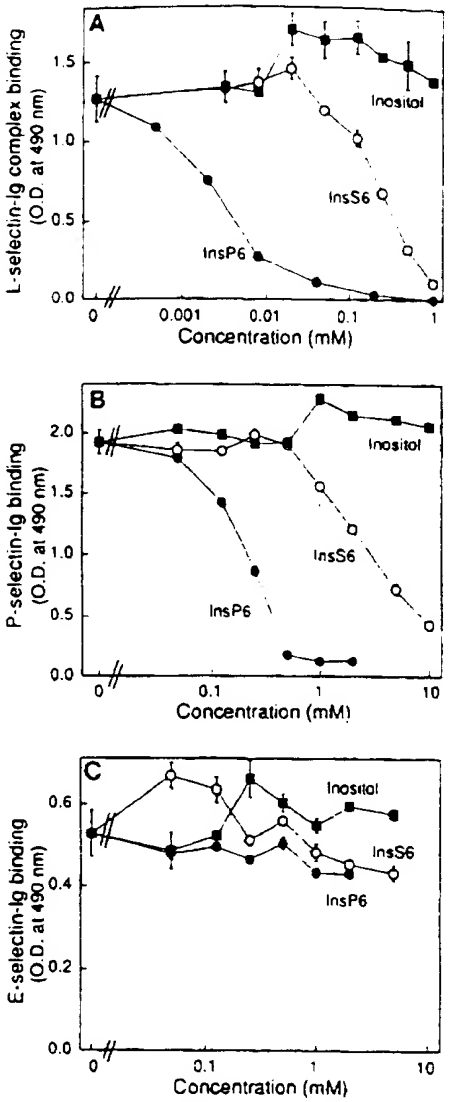

FIG. 2. **Effect of *myo*-inositols on the binding of L- (*a*), P- (*b*), and E-selectin-Ig (*c*) to immobilized BSA-sLe^x in a competition ELISA.** To determine inhibitory activity, $InsP_6$ (●), $InsS_6$ (○), or inositol (■) was added to the selectin-Igs to achieve the indicated concentrations before transfer to microtiter plates. Data shown are the mean and range of optical density measurements made in duplicate wells from a single experiment corrected for background signal, representative of 3–4 separate experiments.

$InsP_6$ and $InsS_6$ Block Cell Adhesion to L- and P-selectin-Ig and to COS Cells Expressing L-and P-selectin—The effect of inositol polyanions on selectin-dependent cell adhesion was as-

TABLE I
Effect of inositol polyanions, inositol, and polyphosphates on L- and P-selectin-Ig binding to BSA-sLe[a]

Compound	L-selectin-Ig		P-selectin-Ig	
	IC_{50}[a]	n[b]	IC_{50}	n
	μM		μM	
$InsP_6$	2.1 ± 1.5	4	160 ± 40	4
$Ins(1,3,4,5,6)P_5$	1.4 ± 0.2	3	260 ± 40	3
D-$Ins(3,5,6)P_3$	340 ± 60	3	NC[c]	2
D-$Ins(1,4,5)P_3$	320 ± 110	3	NC	2
$GroPIns(4,5)P_2$	150 ± 40	3	NC	2
GroPIns	NI[d]	2	NI	2
$InsP_1$	NI	2	NI	2
Inositol	NI	4	NI	3
$InsS_6$	210 ± 80	3	2760 ± 890	3
$Glc(2,3,4,6)P_4$	NI	2	NI	2
Tripolyphosphate	NI	3	NI	3
Trimetaphosphate	NI	3	NI	3
2,3-bisphosphoglycerate	NI	2	NI	2

[a] Concentration of compound that resulted in 50% inhibition of L- or P-selectin-Ig binding to BSA-sLe[a] as measured in a competition ELISA (see "Materials and Methods"). Data represent the mean ± S.D. of the IC_{50} values obtained in n experiments.
[b] n, number of titration experiments performed for each compound.
[c] NC, not calculated (less than 30% inhibition at 2 mM).
[d] NI, no inhibition (at 2 mM).

sessed using a colon cancer cell line, LS180, that binds to all three selectins.[2] As shown in Fig. 3, $InsP_6$ blocked the adhesion of LS180 cells to plates coated with purified L- and P-selectin-Ig (51 ± 5 and 53 ± 14% inhibition at 60 μM, respectively, 5 experiments) but not with E-selectin-Ig. $InsS_6$ was also effective in blocking LS180 adhesion to L- and P-selectin-Ig but required higher concentrations (43 ± 1 and 45 ± 5% inhibition at 4 mM, respectively, 2 experiments); *myo*-inositol, and 2,3-bisphosphoglycerate were inactive at concentrations up to 5 mM. The effect of inositol polyanions on cell adhesion to full-length transmembrane selectins was examined using COS cells transfected with cDNAs encoding L-, P-, and E-selectin (L-COS, P-COS, and E-COS, respectively). $InsP_6$ displayed a dose-dependent inhibition of LS180 cell adhesion to L-COS and P-COS (blocking at 500 μM was 81 ± 13% and 95 ± 5%, respectively, 2 experiments) but not to E-COS (<5% inhibition). In separate studies, PMN adhered to P-COS and E-COS but showed little binding to L-COS. As depicted in Fig. 4, $InsP_6$ (500 μM) blocked PMN adhesion to P-COS but not to E-COS; similar results were obtained with the promyelocytic cell line HL60 (not shown). Higher concentrations of $InsS_6$ also inhibited PMN adhesion to P-COS (63 ± 7% blocking at 5 mM, 2 experiments) but not to E-COS; inositol had no effect at concentrations up to 10 mM.

$InsP_6$ Inhibits PMN Adhesion to Activated Endothelial Cell Monolayers under Fluid Shear Stress—The effect of inositol polyanions on PMN adhesion to cytokine-activated endothelial monolayers was studied under static and nonstatic conditions. In the absence of fluid shear force (static assay), PMN adhesion to tumor necrosis factor α-activated endothelial cells is inhibited by anti-E-selectin antibodies (40) but not by anti-L-selectin antibodies (25). Under these conditions, $InsP_6$ (500 μM) had little or no effect on PMN adhesion (Fig. 5, *upper panel*). In the presence of rotation-induced shear stress (nonstatic assay), PMN adhesion can be inhibited by both anti-E-selectin and anti-L-selectin antibodies (25). Under these conditions, $InsP_6$ (500 μM) reduced PMN adhesion to tumor necrosis factor α-activated endothelial cells to a similar extent as did anti-L-selectin antibodies (Fig. 5, *lower panel*). Inositol was inactive at concentrations up to 10 mM in both static and nonstatic assays.

[2] G. Mannori, L. Carter, O. Cecconi, K. Hanasaki, C. Corless, A. Ariffo, R. M. Nelson, and M. P. Bevilacqua, manuscript in preparation.

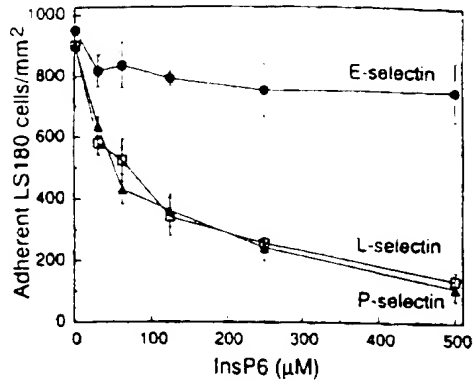

FIG. 3. **Effect of $InsP_6$ on the adhesion of LS180 colon cancer cells to immobilized L- (□), P- (▲), and E-selectin-Ig (●).** $InsP_6$ was added to wells precoated with protein-A-captured selectin-Ig to give the indicated final concentrations. Data presented are mean number of adherent cells/mm² ± S.E., counted in quadruplicate wells in a single experiment representative of three. Inositol was inactive up to 10 mM on each of the selectins. $InsS_6$ inhibited L- and P-selectin-dependent cell adhesion at higher concentrations (43 ± 1 and 45 ± 5% inhibition at 4 mM). No LS180 cell adhesion was detected on plates coated with protein A alone or on plates coated with protein A-captured CD8-Ig. LS180 cell adhesion to E-, P-, and L-selectin was inhibited by blocking monoclonal antibodies H18/7, G1, and LAM1.3, respectively.[2]

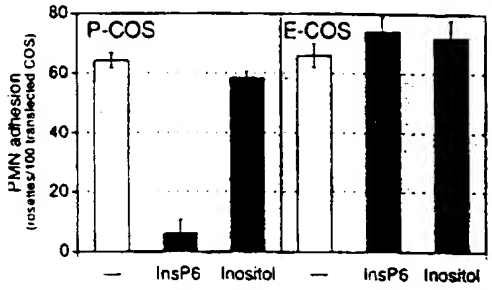

FIG. 4. **Effect of $InsP_6$ or inositol on the adhesion of isolated human PMN to COS cells transfected with P- and E-selectin.** Experiments were conducted as described under "Materials and Methods." Inositol and $InsP_6$ final concentration was 500 μM. Previous experiments showed that the blocking effect was dose-dependent and maximal at concentrations between 250 and 500 μM. Data represent the mean ± S.E. of three separate experiments (five microscopic fields/coverslip with triplicate coverslips were counted in each experiment). Inositol was inactive up to 10 mM. $InsS_6$ inhibited PMN adhesion to P-COS at concentrations higher than 1 mM (63 ± 7% inhibition at 5 mM). Similar results were obtained with HL60 cells.

$InsP_6$ Reduces PMN Accumulation in Experimental Inflammation in Vivo—In mice, peritoneal inflammation induced by injection of thioglycollate results in the accumulation of PMN, the early phase of which is thought to depend largely on L- (45, 46) and P-selectin (47). In this model, $InsP_6$ reduced PMN accumulation in a dose-dependent fashion. Two intravenous injections of 40 μmol/kg resulted in a 55 ± 10% reduction in PMN recovered from the peritoneal cavity at 120 min (Fig. 6*A*). $InsP_5$ (two intravenous injections of 40 μmol/kg) also reduced peritoneal influx of PMN in thioglycollate-stimulated mice (48 ± 6% inhibition, 2 experiments, $p < 0.01$ in both experiments). A single subcutaneous injection of $InsP_6$ (1–160 μmol/kg) acted in a dose-dependent fashion to reduce the number of PMN recovered in the peritoneal cavity of thioglycollate-stimulated animals. The inhibition was maximal between 40 and 160 μmol/kg (45 ± 5% at 40 μmol/kg, 3 experiments). The number of peripheral blood PMN was not reduced by intravenous or subcutaneous $InsP_6$ treatment (not shown). In a closely related murine model, zymosan-induced peritoneal inflammation (Fig. 6*B*), $InsP_6$ (two intravenous injections of 40 μmol/kg at 40 and 80 min) was found to be as effective as in thioglycollate-induced

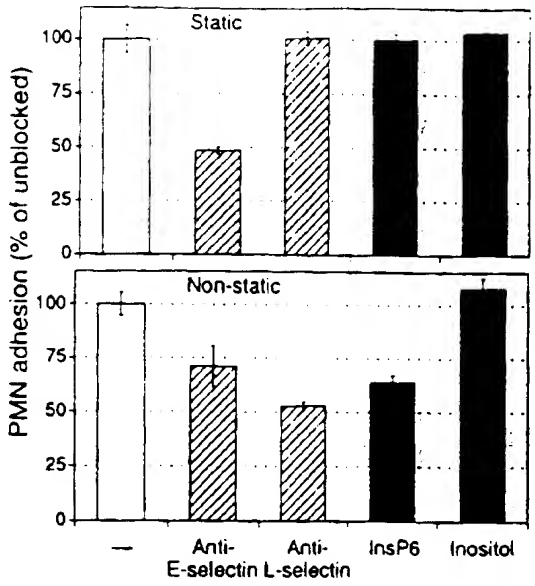

FIG. 5. **Effect of $InsP_6$, inositol, anti-L-selectin, and anti-E-selectin monoclonal antibodies on the adhesion of isolated human PMN to activated endothelial monolayers.** PMN adhesion assay was performed under static (*upper panel*, nonrotating platform) and nonstatic conditions (*lower panel*, rotatory platform, 64 rpm) as described in the text. Inositol and $InsP_6$ concentration was 500 μM. Previous experiments indicated that maximal blocking activity was attained between 250 and 500 μM for $InsP_6$, while inositol was inactive up to 10 mM. Anti-L-selectin antibody (LAM 1.3) was used at a dilution of 1:3000, and anti-E-selectin antibody (H18/7) was used at 10 μg/ml. Data represents the mean ± S.E. of three separate experiments. Unblocked PMN adhesion to activated endothelial monolayers under static and nonstatic conditions ranged from 1800 to 2400 cells/mm^2; unblocked adhesion to unactivated endothelium was less than 50 cells/mm^2.

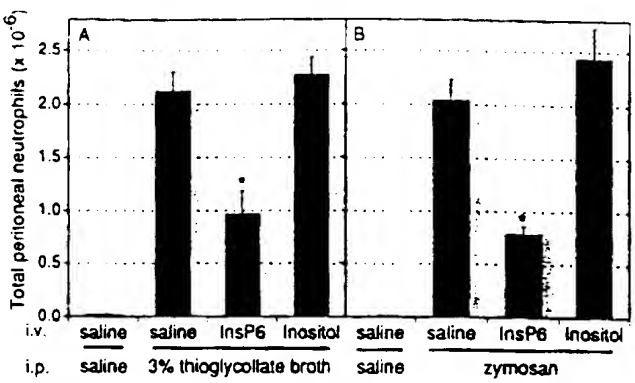

FIG. 6. **Effect of $InsP_6$ on neutrophil accumulation in the peritoneal cavities of mice in two models of acute peritoneal inflammation.** Mice were injected intraperitoneally with 1 ml of 3% thioglycollate broth (Fig. 6*A*) or with 1 ml of saline containing 0.5 mg/ml of zymosan (Fig. 6*B*). Control animals were injected intraperitoneally with 1 ml of sterile pyrogen-free saline. After 40 and 80 min the animals received intravenous injections of 0.2 ml of saline with or without $InsP_6$ (total of 80 μmol/kg = 73 mg/kg) or inositol (400 μmol/kg = 72 mg/kg). Mice were sacrificed after 120 min, and the PMN content of the peritoneal washings was evaluated. *Bars* represents the mean ± S.D. of three separate experiments. Experimental groups contained 5–7 animals/experiment; negative control group (saline intraperitoneally), contained 2 animals/experiment. *Asterisks* indicate statistically significant ($p < 0.01$, Student's t test) decrease in the number of PMN recovered in the peritoneal lavage compared with the positive control group (injected intraperitoneally with thioglycollate or zymosan and intravenously with saline). Peripheral blood PMN and platelet counts were similar between $InsP_6$- and saline-treated groups (not shown). Endotoxin content of injected solutions was less than 0.1 endotoxin units/ml. Treatment of animals with intravenous injections of endotoxin (up to 1 endotoxin unit/ml in saline) did not reduce the number of PMN recovered in the peritoneal washings (not shown).

inflammation (61 ± 4% inhibition, 3 experiments). $InsS_6$ at the same dose did not reduce PMN accumulation in these murine models. However, higher doses (two intravenous injections of 200 μmol/kg) were partially effective yielding a 33 ± 13% inhibition in the thioglycollate model and a 31 ± 11% in the zymosan model (1 experiment each, $p < 0.05$ in both experiments). In a rat model of lung inflammation induced by intratracheal injection of endotoxin (44), $InsP_6$ (two intravenous injections of 20 μmol/kg at 2 and 4 h) reduced the number of PMN recovered in the BAL fluid (56 ± 9% inhibition, $p < 0.01$, 2 experiments). The number of peripheral blood PMN was similar in $InsP_6$- and saline-treated animals (not shown).

DISCUSSION

Inositol polyanions were found to be effective inhibitors of L- and P-selectin *in vitro*. Notably, solution-phase $InsP_5$ and $InsP_6$ blocked the binding of purified L-selectin-Ig fusion protein to BSA-sLe^x at a concentration of 1–2 μM. By comparison, solution-phase sLe^x, a known ligand for this selectin, is far less potent, requiring concentrations in excess of 5 mM to achieve comparable blocking (2, 19, 29). Inositol polyanions were also shown to block P-selectin but not E-selectin interactions with BSA-sLe^x. It has been demonstrated previously that solution-phase sLe^x is a relatively good blocker of E-selectin (IC_{50}, ~750 μM) (6). The inhibitory effects of inositol phosphates on L- and P-selectin appear to depend on the number of phosphate groups. $InsP_6$ and $InsP_5$ were most active, $InsP_3$ showed less activity, while $InsP_1$ and inositol itself showed no activity at concentrations up to 5 mM. In preliminary experiments, additional synthetic isomers of $InsP_3$ showed different inhibitory activity (not shown) suggesting the possibility that the position of the phosphate groups in the inositol ring may be important. Other polyphosphates including glucose 2,3,4,6-tetrakisphosphate, 2,3-bisphosphoglycerate, sodium trimetaphosphate, and sodium tripolyphosphate were inactive up to concentrations of 2 mM. Interestingly, the inhibitory effects of inositol polyanions on L- and P-selectin but not E-selectin parallel the recently described effects of heparin, a polysulfated polysaccharide (6, 21, 22, 29). In particular, both high molecular weight heparin (6, 21, 22) and heparin oligosaccharides (29) have been shown to inhibit the function of L- and P-selectin but not E-selectin. In separate studies, endothelial heparin-like molecules have been suggested as ligands for L-selectin (28). Although several proteins are able to bind inositol polyanions, their affinities for the different polyanions vary. For example, hemoglobin (48) and acidic fibroblast growth factor (49, 50) bind $InsP_6$ more avidly than $InsS_6$, whereas β-adrenergic receptor kinase is inhibited more effectively by $InsS_6$ than by $InsP_6$ (51). In our studies, $InsP_6$ and $InsP_5$ are substantially more active than $InsS_6$ in blocking L- and P-selectin.

Recent studies have provided key insights regarding the carbohydrate recognition domains of C-type lectins, including the selectins. In particular, crystallographic studies on the mannose-binding protein (a C-type lectin homologous to selectins) have revealed that two calcium ions associated with the lectin domain participate in ligand binding (52). In addition, directed mutagenesis and monoclonal antibody mapping of E- and P-selectin have suggested that certain basic amino acids within the lectin domains are important for sLe^x binding (53–55). Inositol polyanions are known to bind basic amino acids in several proteins (56) including hemoglobin (48, 57). It is interesting to speculate that the array of negative charge imparted by phosphate or sulfate groups could facilitate the binding of inositol polyanions to basic amino acids within the selectins. It is also possible that the binding of inositol polyanions to calcium ions within the lectin domain could contribute to their ability to alter selectin function.

Selectins are thought to play a critical role in inflammation by controlling the initial attachment of leukocytes to activated vascular endothelium (58, 59). Previous studies in mice have suggested that L- and P-selectin are involved in the early stages of thioglycollate-induced peritoneal inflammation (45–47). PMN recruitment in this model can be reduced by treatment of the animals with the anti-murine L-selectin monoclonal antibody (Mel-14) (45) or with L-selectin-Ig fusion proteins (46). In addition, P-selectin-deficient mice generated by embryonic stem cell gene inactivation displayed a reduced and delayed appearance of PMN in the peritoneal cavity in response to thioglycollate (47). By using thioglycollate-induced peritoneal inflammation, we showed that $InsP_6$, $InsP_5$, and $InsS_5$, injected intravenously or subcutaneously, can effectively reduce PMN accumulation in the peritoneal cavity of thioglycollate-stimulated animals. The anti-inflammatory activity of $InsP_6$ was also shown in zymosan-induced peritoneal inflammation in mice and endotoxin-induced lung inflammation in rats. Recent observations indicate that oligosaccharide ligands of the selectins are effective blockers of inflammatory responses (16, 18, 29, 60). The present data demonstrate that inositol polyanions, which are low molecular weight noncarbohydrate structures, can also reduce experimental inflammatory responses.

$InsP_6$ and $InsP_5$ are found in substantial quantities in most mammalian cells (61–67), where they may act as precursors of several inositol phosphates thought to be involved in intracellular signaling (68–70). In addition, $InsP_6$ is an abundant constituent of many plant seeds and is found in a variety of foods (56). The possibility that these naturally occurring molecules act to suppress inflammatory or immunological responses deserves attention. In addition to blocking the selectins, inositol polyanions have been shown to possess anti-oxidant activity (71) that could enhance their ability to protect against the tissue damage associated with inflammation (72, 73). It is anticipated that inositol polyanions or their derivatives may prove useful as therapeutic agents in the treatment of a variety of human inflammatory diseases.

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C

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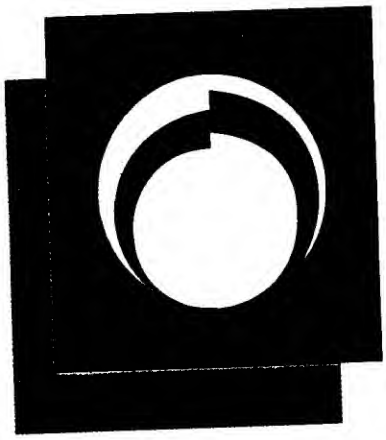

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CHAPTER THREE

Inflammation and Repair

In Chapter 1 we saw how various exogenous and endogenous stimuli can cause cell injury. These same stimuli also can provoke a *complex reaction in the vascularized connective tissue* called inflammation. Invertebrates with no vascular system, single-celled organisms, and multicellular parasites all have their own responses to local injury. These include phagocytosis of the injurious agent; entrapment of the irritant by specialized cells (hemocytes), which then ingest it; and neutralization of noxious stimuli by hypertrophy of the cell or one of its organelles.[1] All these reactions have been retained in evolution, but what characterizes the inflammatory process in higher forms is *the reaction of blood vessels, leading to the accumulation of fluid and leukocytes in extravascular tissues.*

The inflammatory response is closely intertwined with the process of repair. Inflammation serves to destroy, dilute, or wall off the injurious agent, but it, in turn, sets into motion a series of events that, as far as possible, heal and reconstitute the damaged tissue. Repair begins during the early phases of inflammation but reaches completion usually after the injurious influence has been neutralized. During repair, the injured tissue is replaced by *regeneration* of native parenchymal cells, or by filling of the defect with fibroblastic tissue *(scarring)*, or most commonly by a combination of these two processes.

Inflammation is fundamentally a protective response whose ultimate goal is to rid the organism of both the initial cause of cell injury (e.g., microbes, toxins) and the consequences of such injury, the necrotic cells and tissues. Without inflammation, infections would go unchecked, wounds would never heal, and injured organs might remain permanent festering sores. *However, inflammation and repair may be potentially harmful.* Inflammatory reactions, for example, underlie life-threatening hypersensitivity reactions to insect bites, drugs, and toxins as well as some of the common chronic diseases of modern times, such as rheumatoid arthritis, atherosclerosis, and lung fibrosis. Repair by fibrosis may lead to disfiguring scars or fibrous bands that cause intestinal obstruction or limit the mobility of joints. For this reason our pharmacies abound with "anti-inflammatory drugs," which

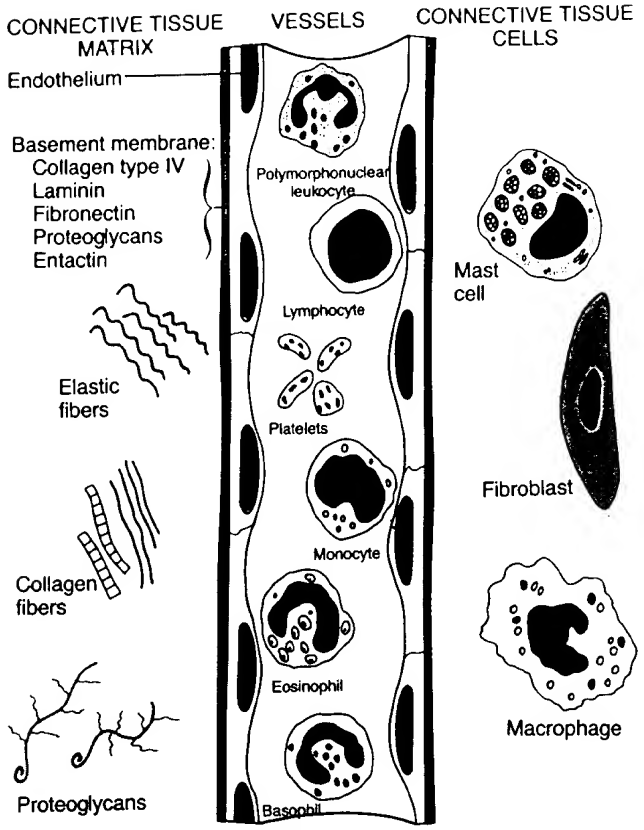


Figure 3–1. Intravascular cells and connective tissue matrix and cells involved in the inflammatory response.

ideally would enhance the salutary effects of inflammation yet control its harmful sequelae.

The inflammatory response occurs in the vascularized connective tissue, including plasma, circulating cells, blood vessels, and cellular and extracellular constituents of connective tissue (Fig. 3–1). The circulating cells include *neutrophils, monocytes, eosinophils, lymphocytes, basophils,* and *platelets.* The connective tissue cells are the *mast cells,* which intimately surround *blood vessels;* the connective tissue *fibroblasts;* and occasional *resident macrophages* and *lymphocytes.* The extracellular matrix (ECM), as described in Chapter 2, consists of the structural fibrous proteins *(collagen, elastin),* the adhesive glycoproteins *(fibronectin, laminin, nonfibrillar collagen, entactin, tenascin,* and others), and proteoglycans. The basement membrane is a specialized component of the ECM consisting of adhesive glycoproteins and proteoglycans.

Inflammation is divided into acute and chronic patterns. *Acute inflammation* is of relatively short duration, lasting for minutes, several hours, or a few days, and its main characteristics are the exudation of fluid and plasma proteins (edema) and the emigration of leukocytes, predominantly neutrophils. *Chronic inflammation,* on the other hand, is of longer duration and is associated histologically with the presence of lymphocytes and macrophages and with the proliferation of blood vessels and connective tissue. Many factors modify the course and histologic appearance of both acute and chronic inflammation, and these will become apparent later in this chapter.

The vascular and cellular responses of both acute and chronic inflammation are mediated by chemical factors derived from plasma or cells and triggered by the inflammatory stimulus. Such mediators, acting singly, in combinations, or in sequence, then *amplify* the inflammatory response and influence its evolution. But it must be remembered that necrotic cells or tissues themsevles—whatever the cause of cell death—can also trigger the elaboration of inflammatory mediators. Such is the case with the acute inflammation following myocardial infarction. Inflammation is *terminated* when the injurious stimulus is removed and the mediators are either dissipated or inhibited.

In this chapter we shall first describe the sequence of events in acute inflammation, as well as the structural and molecular mechanisms underlying them, and then review the various classes of specific mediators that contribute to these events. This will be followed by a discussion of chronic inflammation and then repair, with emphasis on *wound healing* as a classic example of repair. But inflammation has a rich history, intimately linked to the history of wars, wounds, and infections, and we shall first touch on some of the historical highlights in our understanding of this fascinating process.[1,2]

HISTORICAL HIGHLIGHTS

Though they were described in an Egyptian papyrus (3000 BC), *Celsus,* a Roman writer of the first century A.D., first listed the four cardinal signs of inflammation: *rubor, tumor, calor,* and *dolor* (redness, swelling, heat, and pain). A fifth clinical sign, loss of function *(functio laesa),* was later added by Virchow. In 1793, the Scottish surgeon *John Hunter* noted what is now considered an obvious fact: that inflammation is not a disease but a nonspecific response that has a "salutary" effect on its host.[3] *Julius Cohnheim* (1839–1884) first used the microscope to observe inflamed blood vessels in thin, transparent membranes, such as the mesentery and tongue of the frog. Noting the initial changes in blood flow, the subsequent edema due to increased vascular permeability, and the characteristic leukocyte emigration, he wrote descriptions that can hardly be improved on.[4]

The Russian biologist *Elie Metchnikoff* discovered the process of *phagocytosis* by observing the ingestion of rose thorns by amebocytes of starfish larvae and of bacteria by mammalian leukocytes (1882).[5] He concluded that the purpose of inflammation was to bring phagocytic cells to the injured

area to engulf invading bacteria. At that time, Metchnikoff contradicted the prevailing theory that the purpose of inflammation was to bring in factors from the serum to neutralize the infectious agents. It soon became clear that both cellular (phagocytes) and serum factors (antibodies) were critical to the defense against micro-organisms, and in recognition of this both Metchnikoff and Paul Ehrlich (who developed the humoral theory) shared the Nobel Price in 1908.

To these names must be added that of *Sir Thomas Lewis,* who, on the basis of simple experiments involving the inflammatory response in skin, established the *concept that chemical substances, such as histamine locally induced by injury, mediate the vascular changes of inflammation.* This fundamental concept underlies the important discoveries of *chemical mediators* of inflammation and the potential to use anti-inflammatory agents.

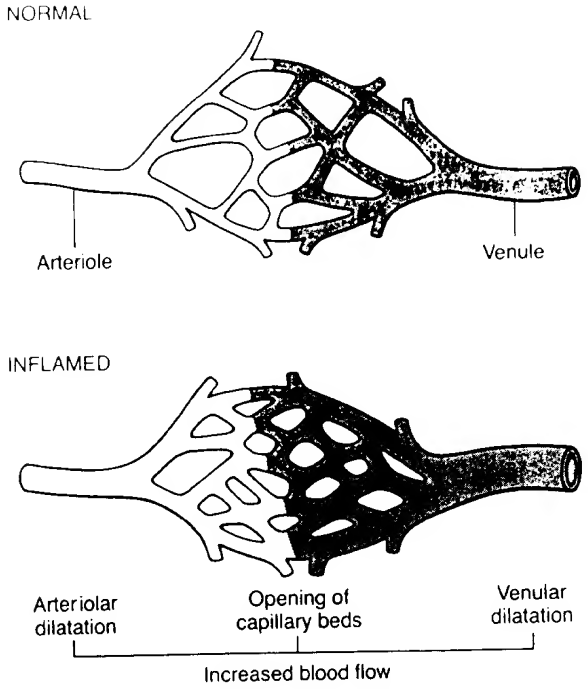


Figure 3–2. Alterations in blood flow associated with inflammation.

ACUTE INFLAMMATION

Acute inflammation is the immediate and early response to an injurious agent. Since the two major defensive components against microbes—antibodies and leukocytes—are normally carried in the bloodstream, it is not surprising that vascular phenomena play a major role in acute inflammation. Therefore, acute inflammation has three major components: *(1) alterations in vascular caliber that lead to an increase in blood flow, (2) structural changes in the microvasculature that permit the plasma proteins and leukocytes to leave the circulation, and (3) emigration of the leukocytes from the microcirculation and their accumulation in the focus of injury.*

Certain terms must be defined before we describe specific features of inflammation. The escape of fluid, proteins, and blood cells from the vascular system into the interstitial tissue or body cavities is known as *exudation.* An *exudate* is an inflammatory extravascular fluid that has a high protein concentration, much cellular debris, and a specific gravity above 1.020. It implies significant alteration in the normal permeability of small blood vessels in the area of injury. In contrast, a *transudate* is a fluid with low protein content (most of which is albumin) and a specific gravity of less than 1.012. It is essentially an ultrafiltrate of blood plasma and results from hydrostatic imbalance across the vascular endothelium. In this situation, the permeability of the endothelium is normal. *Edema* denotes an excess of fluid in the interstitial or serous cavities; it can be either an exudate or a transudate. *Pus,* a *purulent exudate,* is an inflammatory exudate rich in leukocytes (mostly neutrophils) and parenchymal cell debris.

VASCULAR CHANGES

Changes in Vascular Flow and Caliber

Changes in vascular flow and caliber begin very early after injury and develop at varying rates, depending on the severity of the injury. The changes occur in the following order:

- Following an inconstant and transient vasoconstriction of arterioles, lasting a few seconds, *vasodilatation* occurs. This first involves the arterioles and then results in opening of new capillary beds in the area. Thus comes about *increased blood flow,* which is the cause of the heat and the redness (Fig. 3–2). How long vasodilatation lasts depends on the stimulus; it is followed by the next event:
- *Slowing of the circulation.* This is brought about by *increased permeability of the microvasculature,* with the outpouring of protein-rich fluid into the extravascular tissues. The latter results in concentration of red cells in small vessels and increased viscosity of the blood, reflected by the presence of dilated small vessels packed with red cells—termed *stasis.*
- As stasis develops one begins to see peripheral orientation of leukocytes, principally neutrophils, along the vascular endothelium, a process called *leukocytic margination.* Leukocytes then stick to the endothelium, at first transiently (rolling),

then more avidly, and soon afterward they migrate through the vascular wall into the interstitial tissue, in processes that will be discussed presently.

The time scale of these events is variable. With mild stimuli the stages of stasis may not become apparent until 15 to 30 minutes have elapsed, whereas with severe injury, stasis may occur in but a few minutes.

Increased Vascular Permeability (Vascular Leakage)

Increased vascular permeability leading to the escape of a protein-rich fluid (exudate) into the interstitium *is the hallmark of acute inflammation.* The loss of protein-rich fluid from the plasma reduces the intravascular osmotic pressure and increases the osmotic pressure of the interstitial fluid. Together with the increased hydrostatic pressure due to vasodilatation, this leads to a marked *outflow* of fluid and its accumulation in the interstitial tissue (Fig. 3–3). This net increase of extravascular fluid is *edema.*

Normal fluid exchange and microvascular permeability are critically dependent on an intact en-

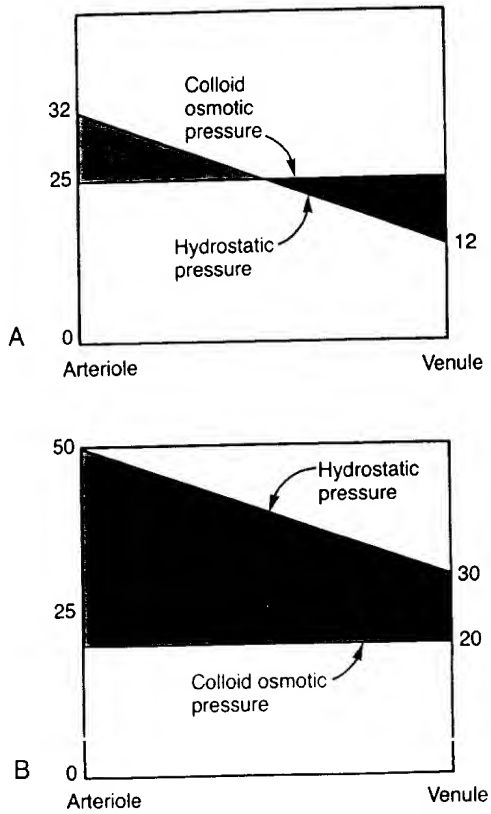


Figure 3–3. Blood pressure and plasma colloid osmotic forces in normal and inflamed microcirculation. *A*, Normal hydrostatic pressure of about 32 mm Hg at arterial end of capillary and 12 mm Hg at venous end. Mean capillary pressure equals colloid osmotic pressure *(horizontal line).* *B*, Acute inflammation. Mean capillary pressure is increased because of arteriolar dilatation, while osmotic pressure is reduced because of protein leakage across venule. Result is net excess of extravasated fluid. (Redrawn with permission from Wright, G.P.: An Introduction to Pathology, 3rd ed. London, Longmans, Green and Co., 1958.)

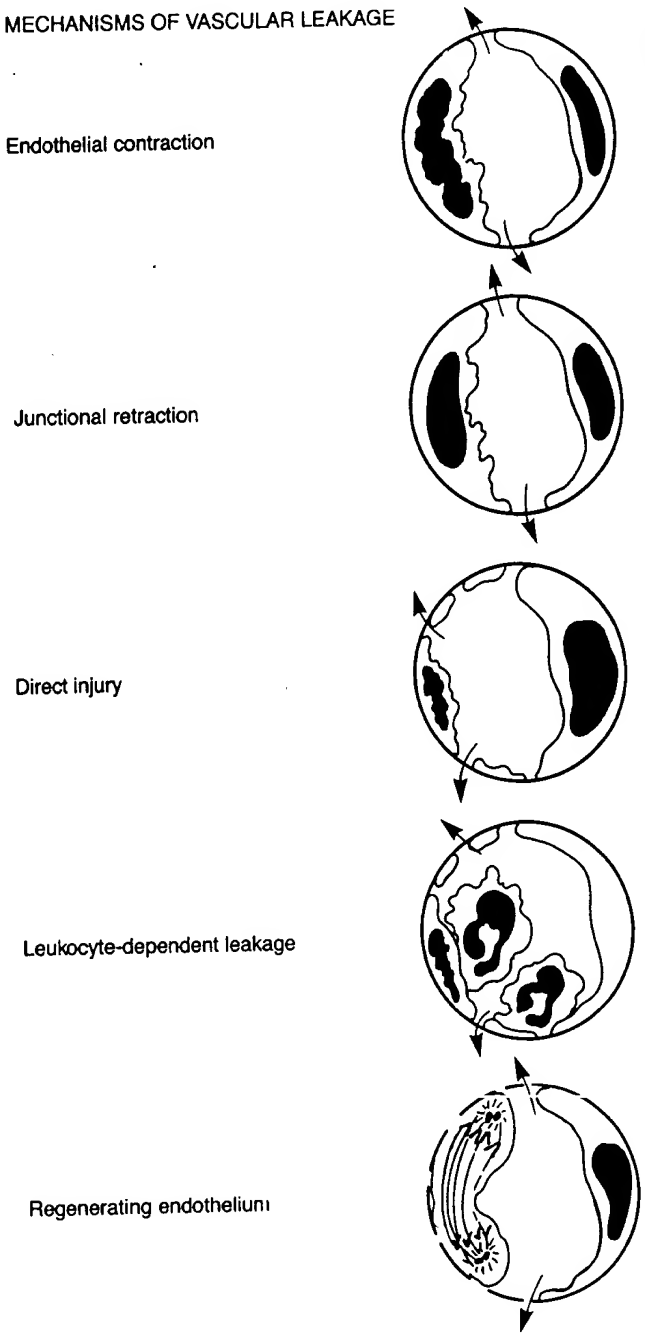


Figure 3–4. Diagrammatic representation of the five mechanisms of increased vascular permeability in inflammation (see text).

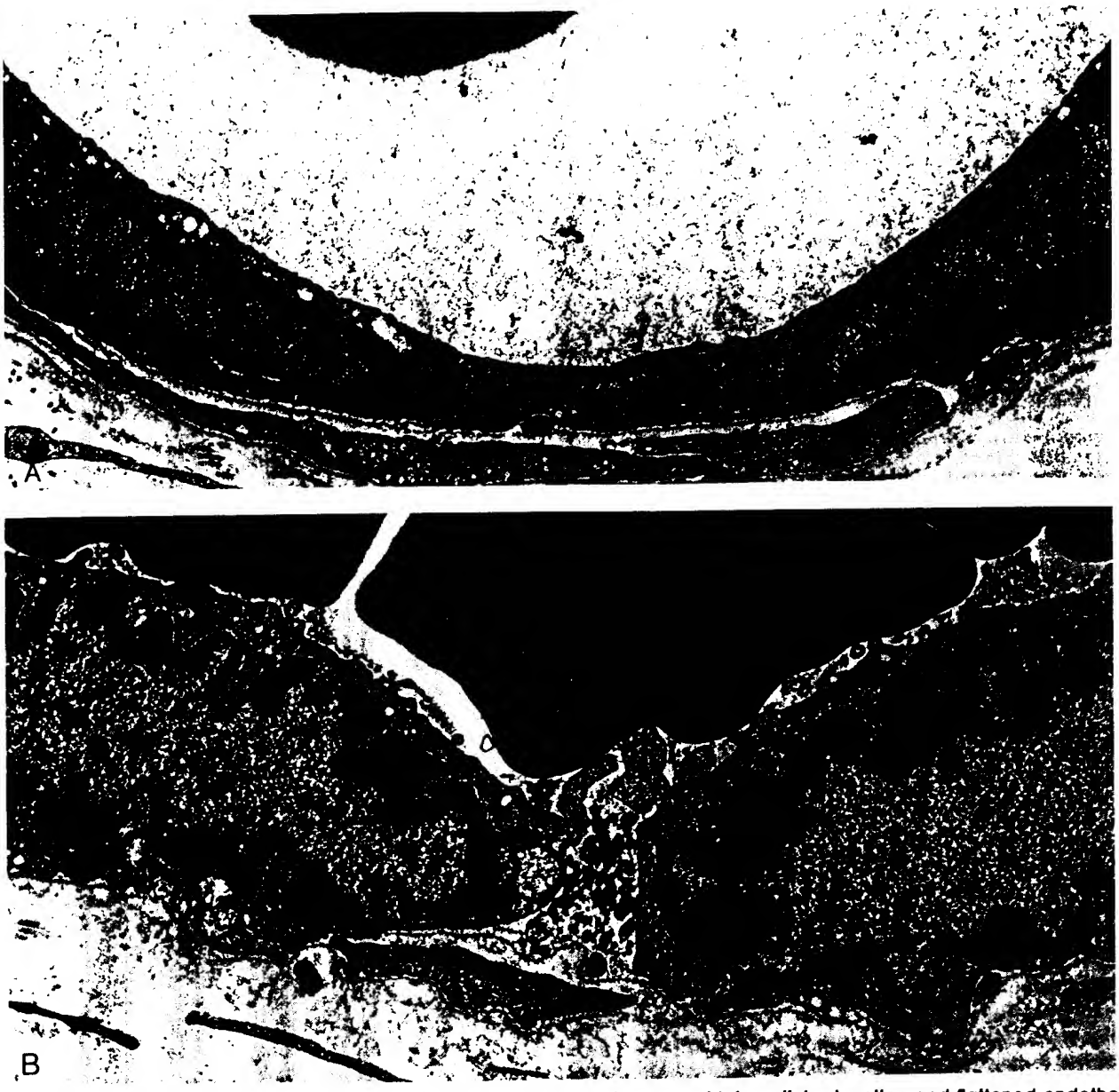


Figure 3–5. *A*, Electron micrograph of wall of normal venule, showing closed intercellular junction and flattened endothelial cells. *B*, Venule after injection of a histamine-type mediator, showing an intercellular gap through which injected black carbon particles have leaked. Note that the cells have bulged into the lumen and their nuclei show many indentations, suggesting contraction. (With permission from Joris, I.J., et al.: The mechanism of vascular leakage induced by leukotriene E_4. Am. J. Pathol. 126:19, 1987.)

dothelium. How then does the endothelium become leaky in inflammation? At least five mechanisms are known (Fig. 3–4).[7]

1. *Endothelial cell contraction, leading to the formation of widened intercellular junctions, or intercellular gaps* (Fig. 3–5).[8] This is by far the most common mechanism of vascular leakage and is elicited by histamine, bradykinin, leukotrienes, and many other classes of chemical mediators. This type of vascular leakage occurs rapidly after exposure to the mediator and is usually reversible and short-lived (15 to 30 minutes); it is thus known as the *immediate transient response.*

Classically, this type of leakage affects only venules 20 to 60 μm in diameter, leaving capillaries and arterioles unaffected (Fig. 3–6). The precise reason for this restriction to venules is uncertain but may be related to a greater density of receptors to the putative mediator in venular endothelium. Parenthetically, many of the later leukocyte events in inflammation—adhesion and emigration—also occur predominantly in the venules in most organs.

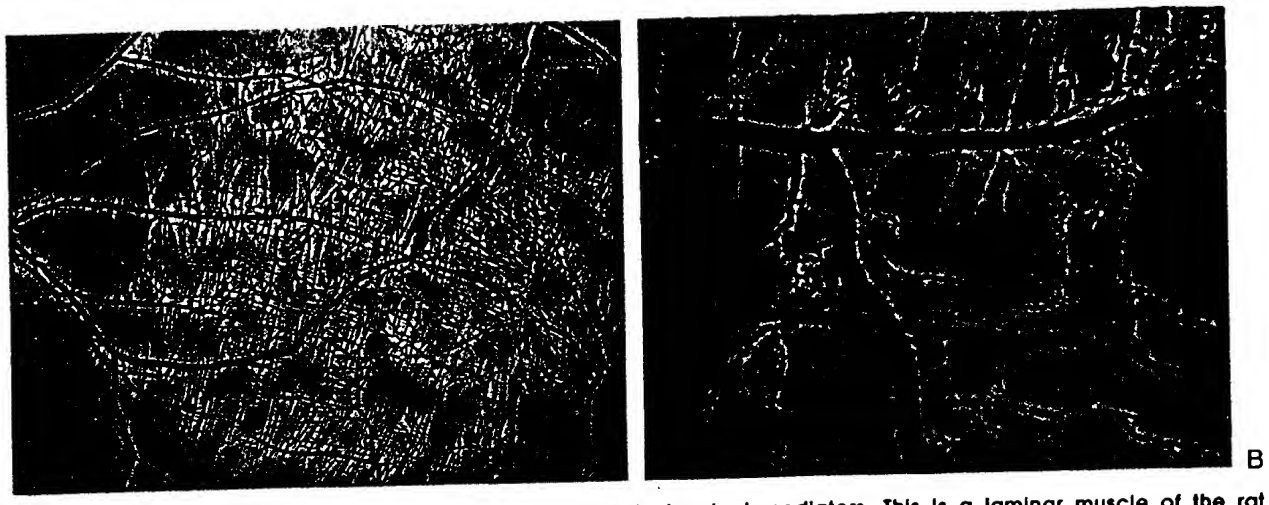


Figure 3–6. *A* and *B*, Vascular leakage as induced by most chemical mediators. This is a laminar muscle of the rat (cremaster), fixed, cleared in glycerin, and examined unstained by transillumination. One hour prior to sacrifice, bradykinin was injected over this muscle, and colloidal carbon was given intravenously; bradykinin caused small gaps to appear between endothelial cells in some vessels. Plasma, loaded with carbon, escaped, but most of the carbon particles were retained by the basement membrane of the leaking vessels, with the result that these became "labeled" in black. *Note that not all the vessels leak*—only the venules. In *B*, a higher power, the capillary network is very faintly visible in the background. (Courtesy of Dr. Guido Majno.)

2. *Cytoskeletal and junctional reorganization (endothelial retraction).* An apparently different mechanism of *reversible* intercellular leakage, resulting in interendothelial gaps, can be induced *in vitro* by cytokine mediators, such as interleukin-1 (IL-1), tumor necrosis factor (TNF), and interferon-gamma (IFN-γ).[9] These cytokines cause a structural reorganization of the cytoskeleton, such that endothelial cells retract from one another along their junctions, leading to endothelial discontinuities. In contrast to the histamine effect, the response is somewhat delayed (4 to 6 hours) and long-lived (24 hours or more). How frequently this mechanism accounts for vascular leakage *in vivo* is still uncertain.

3. *Direct endothelial injury, resulting in endothelial cell necrosis and detachment.* This effect is usually encountered in necrotizing injuries and is due to direct damage to the endothelium by the injurious stimulus, as for example by severe burns or lytic bacterial infections. In most instances leakage starts immediately after injury and is sustained at a high level for several hours until the damaged vessels are thrombosed or repaired. The reaction is known as the *immediate sustained response. All levels of the microcirculation are affected, including venules, capillaries, and arterioles.* Endothelial cell detachment is often associated with platelet adhesion and thrombosis.

Delayed prolonged leakage is a curious but relatively common type of increased permeability that *begins after a delay of 2 to 12 hours, lasts for several hours or even days, and involves venules as well as capillaries.* Such leakage is caused, for example, by mild-to-moderate thermal injury, X- or ultraviolet radiation, and certain bacterial toxins. The late-appearing sunburn is a good example of a delayed reaction. The mechanism for such leakage is unclear. It may result from the direct effect of the injurious agent, leading to delayed cell damage (perhaps by apoptosis), or the effect of cytokines causing endothelial retraction, as described earlier.

4. *Leukocyte-mediated endothelial injury.* Leukocytes adhere to endothelium relatively early in inflammation. As we shall see, such leukocytes may be activated in the process, releasing toxic oxygen species and proteolytic enzymes, which then cause endothelial injury or detachment—resulting in increased permeability.

5. *Leakage from regenerating capillaries.* As described later in this chapter, during repair, endothelial cells proliferate and form new blood vessels *(angiogenesis)*. These capillary sprouts remain leaky until the endothelial cells differentiate and form intercellular junctions, accounting for the edema characteristic of healing inflammation.

Increased transcytosis via vesicles and vacuoles across the cytoplasm is another potential mechanism of increased permeability and has been demonstrated in blood vessels within tumors.[9a]

It should be noted that although these mechanisms are separable, all may play a role in response to one stimulus. For example, in various stages of a thermal burn, leakage results from chemically mediated endothelial contraction as well as direct and leukocyte-dependent injury—and from regenerating capillaries when the injury heals. This accounts for the life-threatening loss of fluid in severely burned patients.

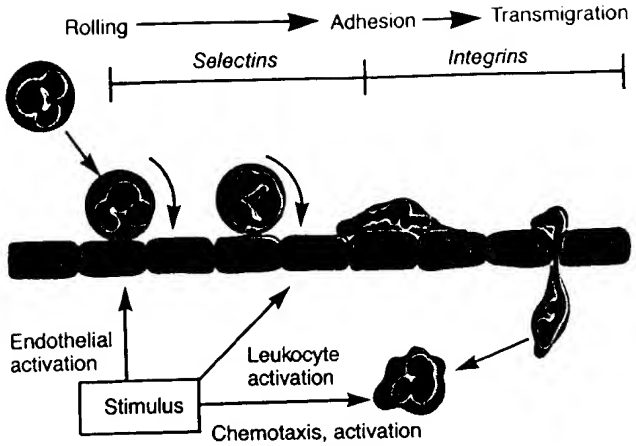


Figure 3–7. Sequence of leukocytic events in inflammation. The leukocytes first *roll*, then arrest and adhere to endothelium, then transmigrate through an intercellular junction, pierce the basement membrane, and migrate toward chemoattractants emanating from the source of injury. The roles of selectins, activating agents, and integrins are also shown. (Modified and redrawn from Travis, J.T.: Biotech gets a grip on cell adhesion. Science 26:906, 1993.)

CELLULAR EVENTS: LEUKOCYTE EXTRAVASATION AND PHAGOCYTOSIS

A critical function of inflammation is the delivery of leukocytes to the site of injury. Leukocytes ingest offending agents, kill bacteria and other microbes, and degrade necrotic tissue and foreign antigens. Unfortunately, leukocytes may also prolong inflammation and induce tissue damage by releasing enzymes, chemical mediators, and toxic oxygen radicals.

The sequence of events in the journey of leukocytes from the lumen to the interstitial tissue, called extravasation, can be divided into the following steps: (1) in the lumen: margination, rolling, and adhesion; (2) transmigration across the endothelium (also called diapedesis); and (3) migration in interstitial tissues toward a chemotactic stimulus (Fig. 3–7).

In normally flowing blood, erythrocytes and leukocytes are confined to a central axial column, leaving a cell-poor layer of plasma in contact with endothelium. As blood flow slows early in inflammation (as a result of the increased vascular permeability), white cells fall out of the central column and assume a peripheral position along the endothelial surface. This initial process, called *margination*, is caused largely by changes in hemodynamic conditions engendered by low and slow blood circulation. Subsequently, individual and then rows of leukocytes tumble slowly along the endothelium and adhere transiently (a process called *rolling*), finally coming to rest at some point where they adhere firmly (resembling "pebbles or marbles over which a stream runs without disturbing them"). In time, the endothelium can be virtually lined by white cells, an appearance called "*pavementing.*" Following firm adhesion, leukocytes insert pseudopods into the junctions between the endothelial cells, squeeze through interendothelial junctions, and assume a position between the endothelial cell and the basement membrane. Eventually they traverse the basement membrane and escape into the extravascular space. Neutrophils, monocytes, lymphocytes, eosinophils, and basophils all use the same pathway. We shall now examine the molecular mechanisms of each of the steps.

Adhesion and Transmigration

It is now clear that leukocyte adhesion and transmigration are determined largely by the binding of complementary adhesion molecules on the leukocyte and endothelial surfaces (like a key and lock), and that chemical mediators—chemoattractants and certain cytokines—affect these processes by modulating the surface expression or avidity of such adhesion molecules.[6,10]

The adhesion receptors involved belong to three molecular families—the *selectins*, the *immunoglobulins*, and the *integrins*. The most important of these are shown in Table 3–1.[11]

Selectins, so-called because they are characterized by an extracellular N-terminal domain related to sugar-binding mammalian lectins, consist of E-selectin (also known as ELAM-1) that is confined to endothelium; P-selectin (also called GMP140), present in endothelium and platelets; and L-selectin (also called LAM-1), which decorates most leukocyte types. P and E selectins bind, through their lectin domain, to sialylated forms of oligosaccharides (e.g., sialylated Lewis X), which themselves are covalently bound to various cell surface glycoproteins. L-selectin binds to mucin-like glycoproteins (GlyCAM-1 and CD34).

The *immunoglobulin* family molecules include two *endothelial* adhesion molecules: ICAM-1 (intercellular adhesion molecule 1) and VCAM-1 (vascular cell adhesion molecule 1), and both interact with *integrins* found on leukocytes. (You may recall from Chapter 2 that integrins are transmembrane-adhesive heterodimeric glycoproteins, made up of alpha and beta chains that also function as receptors for the extracellular matrix [ECM].) The principal integrin receptors for ICAM-1 are the β_2 integrins LFA-1 and MAC-1 (CD11a/CD18 and CD11b/CD18), and that for VCAM-1 is the β_1 integrin VLA-4 ($\alpha_4\beta_1$ integrin).

But how are these molecules modulated to induce adhesion in inflammation? There are a number of mechanisms, dependent on the duration of

Table 3–1. LEUKOCYTE–ENDOTHELIAL ADHESION MOLECULES

ENDOTHELIAL MOLECULE	*Family*	LEUKOCYTE RECEPTOR	*Family*	*Distribution*
E-selectin (ELAM-1)	Selectin	Sialyl-Le^x Glycoprotein	Oligosaccharides	Neutrophils T cells Monocytes
P-selectin (GMP-140)	Selectin	Sialyl-Le^x Glycoprotein	Oligosaccharides	Neutrophils Monocytes
ICAM-1	Immunoglobulin	LFA-1 Mac-1	β_2 Integrin	All leukocytes
VCAM-1	Immunoglobulin	VLA-4	β_1 Integrin	Lymphocytes Monocytes Basophils Eosinophils
LAM-1 Ligand(s) (GlyCam-1; CD34)	Mucin-like glycoproteins	L-selectin (LAM-1)	Selectin	Neutrophils Lymphocytes Monocytes

Modified and used with permission from Briscoe, D.M., and Cotran, R.S.: Role of leukocyte–endothelial cell adhesion molecules in renal inflammation: In vitro and in vivo studies. Kidney Int. 42:S-28, 1993.

inflammation, the type of inflammatory stimulus, and blood flow conditions (Fig. 3–8).

1. *Redistribution of adhesion molecules to the cell surface* (Fig. 3–8A). P-selectin, for example, is normally present in the membrane of specific intracytoplasmic endothelial granules, called Weibel-Palade bodies. On stimulation by mediators such as histamine, thrombin, and platelet-activating factor (PAF), P-selectin is rapidly redistributed to the cell surface, where it can bind the leukocytes.[12] This process occurs within minutes in flowing blood and serves to deliver preformed adhesion molecules in short order to the surface. Studies suggest that this process may be particularly important in early leukocyte *rolling* on endothelium.

2. *Induction of adhesion molecules on endothelium.* Some inflammatory mediators, and particularly cytokines (IL-1 and TNF), induce the synthesis and surface expression of endothelial adhesion molecules (Fig. 3–8B). This process requires new protein synthesis and begins usually after a delay of some 1 or 2 hours.[13] E-selectin, for example, which is not present in normal endothelium, is induced by IL-1 and TNF, maximally after 4 to 6 hours and mediates the adhesion of neutrophils by binding to its receptor sialylated Lewis X. The same cytokines also increase the expression of ICAM-1 and VCAM-1, which are present at low levels in normal endothelium.

3. *Increased avidity of binding* (Fig. 3–8C). This mechanism is most relevant to the binding of integrins. For example, LFA-1 is normally present on leukocytes—neutrophils, monocytes, and lymphocytes—but does not adhere to its ligand ICAM-1 on endothelium. When neutrophils are activated by chemotactic agents or other stimuli, the LFA-1 is converted from a state of low- to high-affinity binding toward ICAM-1, owing to a conformational change in the molecule. During inflammation, the increased affinity of LFA-1 on the activated leukocyte, coupled with the increased ICAM-1 expression on endothelium induced by cytokines, sets the stage for strong LFA-1/ICAM-1 binding. In the low-flow conditions generated by stasis, the LFA-1/ICAM-1 interaction causes *firm adhesion* to the endothelium and appears also to be necessary for the subsequent *transmigration* across the endothelium.

Based on such studies, a currently popular scenario for neutrophil adhesion and transmigration in acute inflammation postulates the following steps (Fig. 3–9):[15] (1) First, there is initial rapid and relatively loose adhesion that accounts for *rolling*, involving mainly the naturally occurring P- and L-selectins, and, in cytokine-induced endothelium, E-selectin; (2) the leukocytes are then activated, by agents made by endothelium or other cells or emanating from the site of injury, to increase the avidity of their integrins (as in Fig. 3–8C); (3) they then bind stably to endothelium, largely through the β_2 integrin–ICAM-1 pathway, and undergo transmigration. Neutrophils, monocytes, eosinophils, and various types of lymphocytes use different (but overlapping) molecules for adhesion, and their adhesivity can be modulated by the state of activation of the leukocyte and endothelium.[16]

The most telling proof of the importance of adhesion molecules is the existence of clinical genetic deficiencies in the leukocyte adhesion proteins, which are characterized by impaired leukocyte adhesion and recurrent bacterial infections. In *leukocyte adhesion deficiency type 1*, patients have a defect in the biosynthesis of the β_2 chain shared by LFA-1 and Mac-1 integrins.[17] *Leukocyte adhesion deficiency type 2* is caused by absence of sialyl-Lewis X, the ligand for E-selectin, due to a generalized defect in fucose metabolism.[18] In addi-

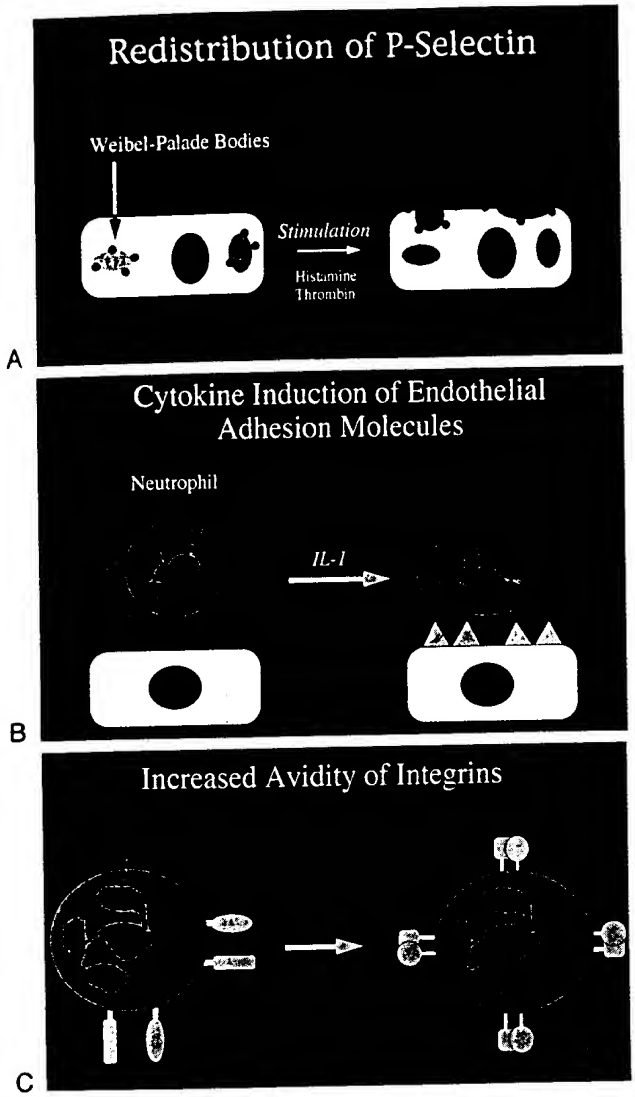


Figure 3–8. Three mechanisms of mediating leukocyte endothelial adhesion. *A*, Redistribution of P-selectin. *B*, Cytokine activation of endothelium. *C*, Increased binding avidity of integrins (see text). (Courtesy of Dr. Madeleine Kraus.)

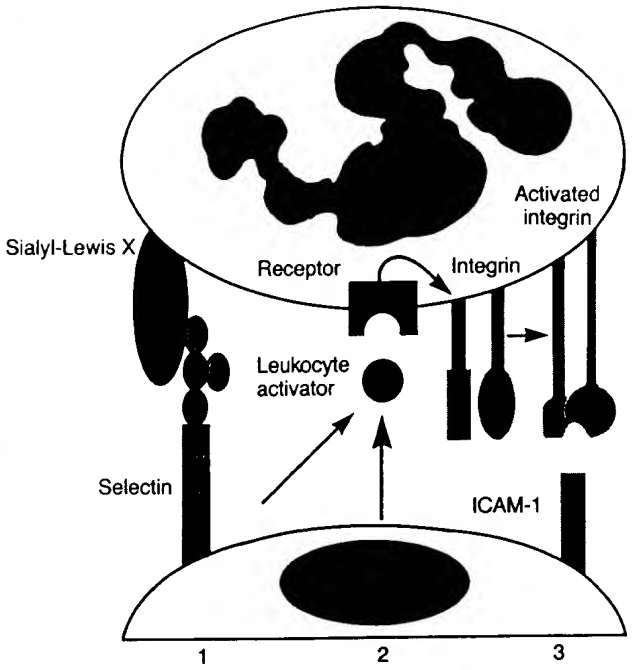


Figure 3–9. Steps in endothelial-neutrophil recognition: (1) initial adhesion via selectins; (2) activation of leukocytes by mediators, or by step 1, causing increased avidity of integrin; and (3) firm adhesion/transmigration via integrin/ICAM-1.

tion, antibodies to adhesion molecules abrogate leukocyte extravasation in experimental models of acute inflammation,[19] and transgenic mice deficient in these molecules show a compromise in leukocyte rolling and extravasation.[20]

As described earlier (see Fig. 3–7), transmigration of all leukocyte types occurs along the intercellular junctions. Certain homophilic adhesion molecules (i.e., adhesion molecules that bind to each other) present in the intercellular junction of endothelium may be involved. One of these is a member of the immunoglobulin gene superfamily called *PECAM-1* (platelet endothelial cell adhesion molecule) or CD31.[21] Antibodies to this molecule inhibit transmigration *in vitro*. In passing, it should be noted that *leukocyte diapedesis, like increased vascular permeability, occurs predominantly in the venules* (except in the lungs, where it also occurs in capillaries). After traversing the endothelial junctions, leukocytes are transiently retarded in their journey by the continuous basement membrane but eventually pierce it, probably by secreting collagenases that degrade the basement membrane.

The type of emigrating leukocyte varies with the age of the inflammatory lesion and with the type of stimulus. In most forms of acute inflammation, *neutrophils predominate in the inflammatory infiltrate during the first 6 to 24 hours, and then are replaced by monocytes in 24 to 48 hours* (Fig. 3–10). The sequence can best be explained by the activation of different adhesion molecule pairs and of chemotactic factors in different phases of inflammation. In addition, short-lived neutrophils disintegrate and disappear after 24 to 48 hours, whereas monocytes survive longer. However, there are exceptions to this pattern of cellular exudation. In certain infections, for example those produced by Pseudomonas organisms, neutrophils predominate over 2 to 4 days; in viral infections, lymphocytes may be the first cells to arrive; in some hypersensitivity reactions, eosinophilic granulocytes may be the main cell type.

Chemotaxis and Leukocyte Activation

Following extravasation, leukocytes emigrate in tissues toward the site of injury by a process called

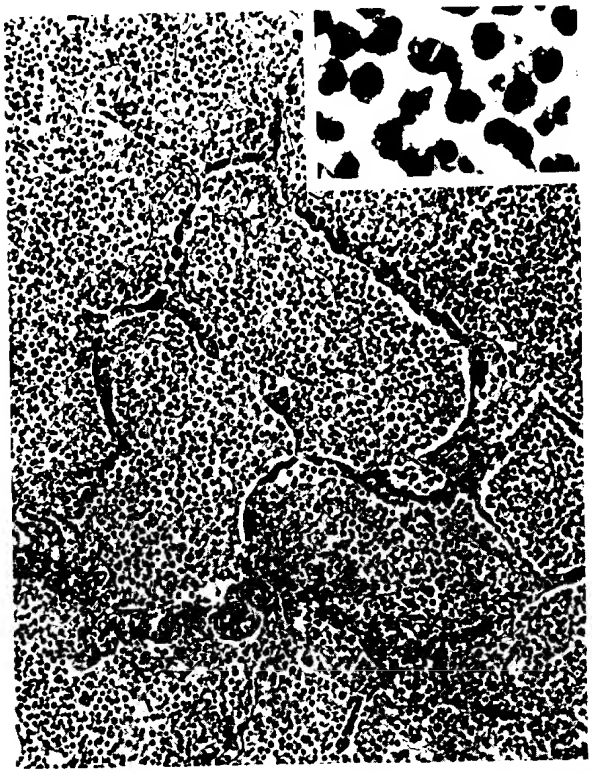


Figure 3–10. Photomicrograph of an acutely inflamed lung (pneumonia) showing emigration of inflammatory cells into the alveoli. Most of the cells in the exudate are neutrophils *(inset)*.

chemotaxis, defined most simply as locomotion oriented along a chemical gradient. All granulocytes, monocytes, and, to a lesser extent, lymphocytes respond to chemotactic stimuli with varying rates of speed.

Both exogenous and endogenous substances can act as chemoattractants. The most common exogenous agents are *bacterial products*. Some of these are peptides that possess an *N*-formyl-methionine terminal amino acid. Others are lipid in nature. Endogenous chemical mediators, which will be detailed later, include (1) *components of the complement system*, particularly C5a, (2) *products of the lipoxygenase pathway*, mainly leukotriene B_4 (LTB_4), and (3) *cytokines*, particularly those of the IL-8 family.

But how does the leukocyte "see" (or "smell") the chemotactic agents, and how do these substances induce directed cell movement? Although not all the answers are known, several important steps and second messengers are recognized (Fig. 3–11).[22] Binding of chemotactic agents to specific receptors on the cell membranes of leukocytes results in activation of phospholipase C (mediated by unique G-proteins), leading to the hydrolysis of phosphatidylinositol-4,5-biphosphate (PIP_2), to inositol-1,4,5-triphosphate (IP_3) and diacylglycerol (DAG), and the release of calcium, first from intracellular stores and subsequently from the influx of extracellular calcium. It is the increased cytosolic calcium that triggers the assembly of contractile elements responsible for cell movement.

The leukocyte moves by extending a pseudopod (lamellipod) that pulls the remainder of the cell in the direction of extension, just as an automobile with front-wheel drive is pulled by the wheels in front (Fig. 3–12). The interior of the pseudopod consists of a branching network of filaments composed of *actin* as well as the contractile protein *myosin*. Locomotion involves rapid assembly of actin monomers into linear polymers at the pseudopod's leading edge, cross-linking of filaments, followed by disassembly of such filaments away from the leading edge.[23] These complex events are controlled by the effects of calcium ions and phosphoinositols on a number of actin-regulating proteins, such as *actin binding protein (filamin)*, *gelsolin*, *profilin*, and *calmodulin*. Precisely how myosin interacts with actin in the pseudopod to produce contraction is unclear.

In addition to stimulating locomotion, many chemotactic factors, particularly in high concentrations, induce other responses in the leukocytes, referred to under the rubric of *leukocyte activation* (see Fig. 3–11). Such responses, which can also be induced by phagocytosis and antigen-antibody complexes, include the following:

- *Production of arachidonic acid metabolites* from phospholipids, due to activation of phospholipase A_2 by DAG and increased intracellular calcium.
- *Degranulation and secretion of lysosomal enzymes, and activation of the oxidative burst* (see discussion under phagocytosis). These two processes are induced by DAG-mediated activation of protein kinase. Activation of intracellular phospholipase D by the increased calcium influx contributes to the sustained DAG accumulation.
- *Modulation of leukocyte adhesion molecules.* Certain chemoattractants cause increased surface expression and, as stated earlier, increased adhesive avidity of the LFA-1 integrin, allowing firm adhesion of activated neutrophils to ICAM-1 on endothelium. In contrast, neutrophils shed L-selectin from their surface, making them less adhesive to the L-selectin ligand on endothelium.

A newly appreciated phenomenon in leukocyte activation is *priming*, denoting an increased rate and extent of leukocyte activation by exposure to a mediator that itself causes little activation. The cytokine TNF, in particular, markedly increases leukocyte activation by other chemotactic agents, accounting for its powerful *in vivo* effects, described later in this chapter.

Phagocytosis

Phagocytosis and the release of enzymes by neutrophils and macrophages constitute two of the

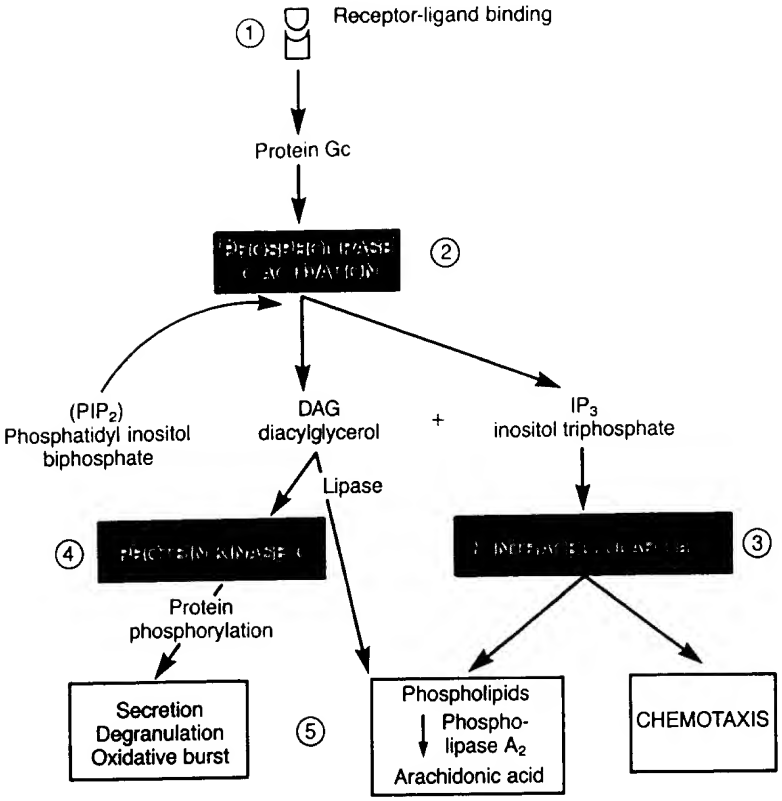


Figure 3–11. Biochemical events in leukocyte activation. The key events are (1) receptor-ligand binding, (2) phospholipase-C activation, (3) increased intracellular calcium, and (4) activation of protein kinase C. The biologic activities (5) resulting from leukocyte activation include chemotaxis, elaboration of arachidonic acid metabolites, secretion, and degranulation. Not shown is phospholipase D activation by increased Ca^{++}, which increases DAG and amplifies protein kinase C activation.

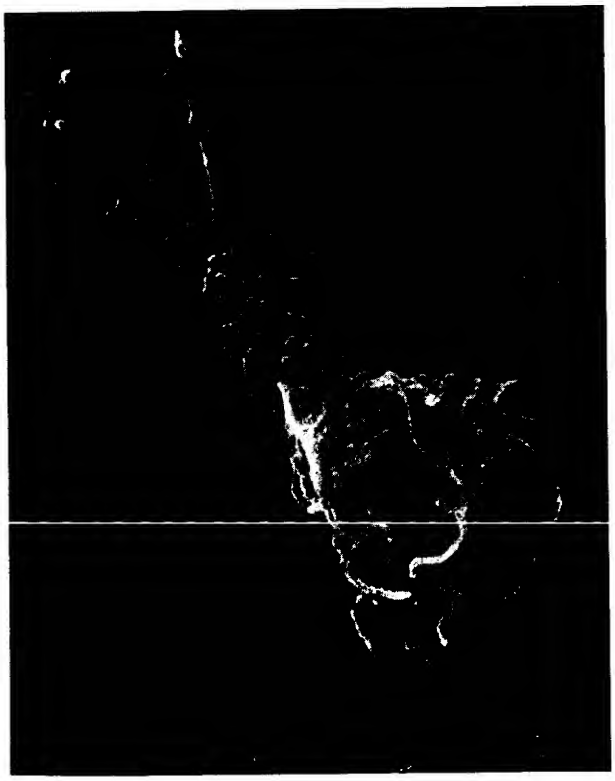


Figure 3–12. Scanning electron micrograph of a moving leukocyte in culture showing a pseudopod *(upper left)* and a trailing tail. (Courtesy of Dr. Morris J. Karnovsky, Harvard Medical School, Boston.)

major benefits derived from the accumulation of leukocytes at the inflammatory focus. Phagocytosis involves three distinct but inter-related steps (Fig. 3–13*A*): *recognition* and *attachment* of the particle to be ingested by the leukocyte; its *engulfment*, with subsequent formation of a phagocytic vacuole; and *killing* or *degradation* of the ingested material.[24]

RECOGNITION AND ATTACHMENT. On occasion neutrophils and macrophages recognize and engulf bacteria or extraneous matter (e.g., latex beads) in the absence of serum. Most microorganisms, however, are not recognized until they are coated by naturally occurring factors called *opsonins*, which bind to specific receptors on the leukocytes. The two major opsonins are (1) the *Fc fragment of immunoglobulin G (IgG)*, presumably naturally occurring antibody against the ingested particle; and (2) *C3b*, the so-called "opsonic fragment of C3" (and its stable form *C3bi*), generated by activation of complement by immune or nonimmune mechanisms, as described later. The corresponding receptors on leukocytes are FcγR which recognize the Fc fragment of IgG, and *complement receptors 1, 2, and 3 (CR1, 2, 3)*, which interact with C3b and C3bi. *CR3*, which recognizes C3bi, is a particularly important receptor; it is identical with the β_2 integrin–Mac-1, which, as you recall, is involved in adhesion to endothelium. It binds certain bacteria by recognizing bacterial lipopolysaccharides (LPS), without the intervention of antibody or complement, accounting for so-called *nonopsonic phagocy-*

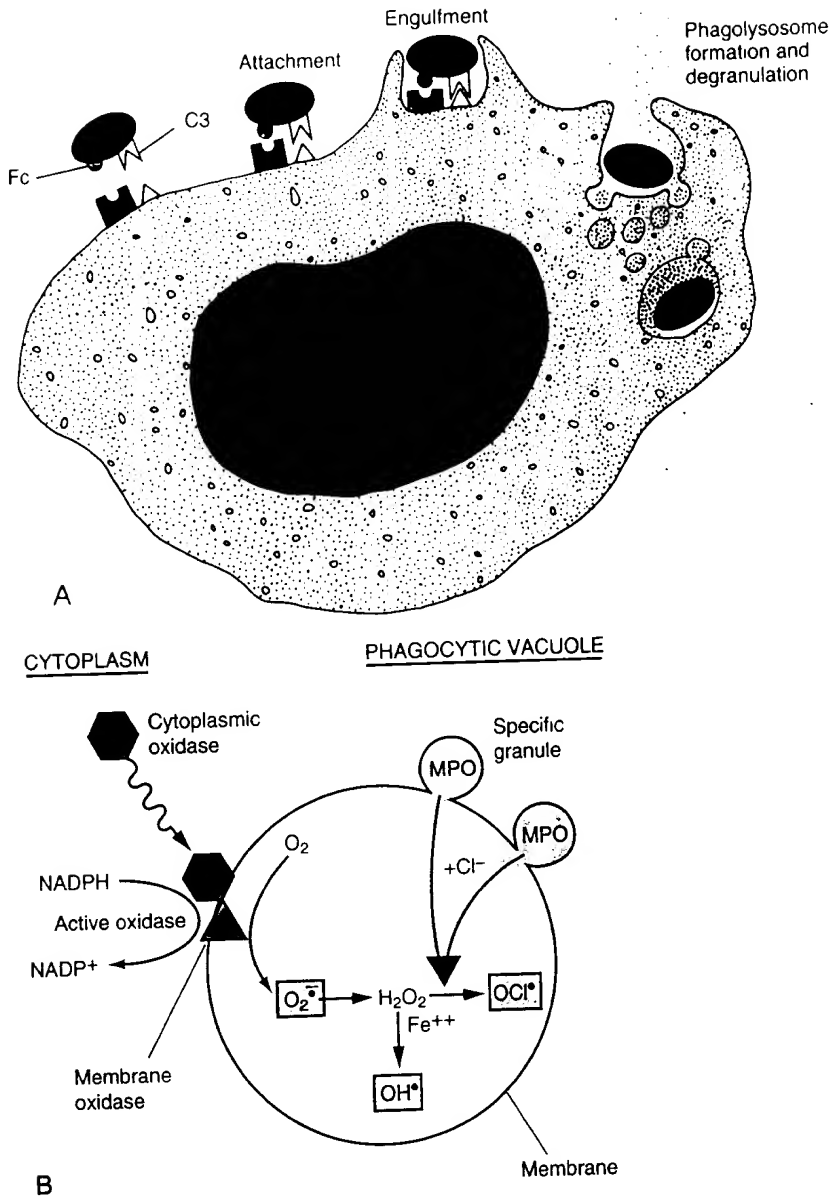


Figure 3–13. *A*, Phagocytosis of a particle (e.g., bacterium) involves attachment and binding of Fc and C3b to receptors on the leukocyte membrane, engulfment, and fusion of granules *(in red)* with phagocytic vacuoles, followed by degranulation. Note that during phagocytosis, granule contents may be released extracellularly. *B*, Summary of oxygen-dependent bactericidal mechanisms within phagocytic vacuole, as described in text.

tosis. CR3 also binds the ECM components fibronectin and laminin.

ENGULFMENT. Binding of the opsonized particle to the FcγR is sufficient to trigger engulfment, which is markedly enhanced in the presence of CRs. However, binding to the C3 receptors alone requires activation of such receptors before engulfment occurs. Such activation is accomplished by simultaneous binding to extracellular fibronectin and laminin or by certain cytokines. During engulfment, extensions of the cytoplasm (pseudopods) flow around the object to be engulfed, eventually resulting in complete enclosure of the particle within a phagosome created by the cytoplasmic membrane of the cell. The limiting membrane of this phagocytic vacuole then fuses with the limiting membrane of a lysosomal granule, resulting in discharge of the granule's contents into the phagolysosome (see Fig. 3–13*A*). In the course of this action, the neutrophil and the monocyte become progressively degranulated.

Many of the biochemical events involved in phagocytosis and degranulation are similar to those described for chemotaxis (see Fig. 3–11). The process is associated with receptor-ligand binding, phospholipase C activation, DAG and IP_3 production, protein kinase C activation, and increased concentration of cytosolic calcium, the latter two acting as second messengers to initiate the cellular events.

KILLING OR DEGRADATION. The ultimate step in phagocytosis of bacteria is killing and degradation. *Bacterial killing is accomplished largely by oxygen-dependent mechanisms* (see Fig. 3–13*B*).[25] Phago-

cytosis stimulates a burst in oxygen consumption, glycogenolysis, increased glucose oxidation via the hexose-monophosphate shunt, and production of reactive oxygen metabolites.

The generation of oxygen metabolites is due to the rapid activation of an oxidase (NADPH oxidase), which oxidizes NADPH (reduced nicotinamide-adenine dinucleotide phosphate) and, in the process, reduces oxygen to superoxide ion (O_2^-).

$$2O_2 + NADPH \xrightarrow{oxidase} 2O_2^- + NADP^+ + H^+$$

Superoxide is then converted into H_2O_2, mostly by spontaneous dismutation ($O_2^- + 2H^+ \longrightarrow H_2O_2$). The NADPH oxidase is a complex enzyme system consisting of *cytosolic* phosphoproteins and *membrane* cytochrome protein components (cytochrome b-558). Activation of the oxidase requires translocation of the cytosolic components to interact with the fixed cytochrome on the membrane or, when the membrane is invaginated, in the phagolysosome (see Fig. 3–13*B*). Thus, the *hydrogen peroxide is produced within the lysosome.*

The quantities of H_2O_2 produced in the phagolysosome are insufficient to induce effective killing of bacteria. However, the azurophilic granules of neutrophils contain the enzyme *myeloperoxidase* (MPO), which, in the presence of a halide such as Cl^-, converts H_2O_2 to $HOCl^*$. The latter is an antimicrobial agent that destroys bacteria by *halogenation* (in which the halide is bound covalently to cellular constituents) or by oxidation of proteins and lipids (lipid peroxidation). A similar mechanism is also effective against fungi, viruses, protozoa, and helminths. Most of the H_2O_2 is eventually broken down by catalase into H_2O and O_2, and some is destroyed by the action of glutathione oxidase.

Although the H_2O_2-MPO-halide system is the most efficient bactericidal system in neutrophils, MPO-deficient leukocytes are capable of killing bacteria (albeit more slowly than control cells), by virtue of the formation of superoxide, hydroxyl radicals, and singlet-oxygen (see Fig. 3–13).

The importance of oxygen-dependent bacterial mechanisms is shown by the existence of a group of congenital disorders in bacterial killing called *chronic granulomatous disease (CGD)*, which make patients susceptible to recurrent bacterial infection.[26] CGD results from *inherited defects in the genes encoding the several components of NADPH oxidase,* which generates superoxide. The most common variants are an *X-linked defect* in one of the subunits of the membrane-bound cytochrome and an *autosomal recessive* defect in gene encoding one of the cytoplasmic components.

Bacterial killing can also occur in the absence of an oxidative burst, by substances in the leukocyte granules. These include *bactericidal permeability increasing protein* (BPI), a highly cationic granule-associated protein that causes phospholipase activation, phospholipid degradation, and increased permeability in the outer membrane of the micro-organisms; *lysozyme,* which hydrolyzes the muramic acid–*N*-acetyl-glucosamine bond, found in the glycopeptide coat of all bacteria; *lactoferrin,* an iron-binding protein present in specific granules; *major basic protein* (MBP), a cationic protein of eosinophils, which has limited bactericidal activity but is cytotoxic to many parasites; and *defensins,* cationic arginine-rich granule peptides that are cytotoxic to microbes (and certain mammalian cells).[27]

Following killing, acid hydrolases found in azurophil granules degrade the bacteria within phagolysosomes. The pH of the phagolysosome drops to between 4 and 5 after phagocytosis, this being the optimal pH for the action of these enzymes.

Release of Leukocyte Products

The membrane perturbations that occur in neutrophils and monocytes during chemotaxis and phagocytosis result in the release of products not only within the phagolysosome but also potentially into the extracellular space. The most important of these substances are (1) *lysosomal enzymes,* present in the neutrophil granules, (2) *oxygen-derived active metabolites,* detailed earlier; and (3) *products of arachidonic acid metabolism,* including prostaglandins and leukotrienes. These products are powerful mediators of endothelial injury and tissue damage and amplify the effects of the initial inflammatory stimulus. Thus, if persistent and unchecked, the leukocytic infiltrate itself becomes the offender, and indeed leukocyte-dependent tissue injury underlies many human diseases, such as rheumatoid arthritis and certain forms of chronic lung disease. This will become evident in the discussion of chronic inflammation.

The ways by which lysosomal granules and enzymes are secreted are diverse. Release may occur if the phagocytic vacuole remains transiently open to the outside before complete closure of the phagolysosome *(regurgitation during feeding).* In cells exposed to potentially ingestible materials, such as immune complexes, on flat surfaces (e.g., glomerular basement membrane), attachment of immune complexes to the leukocyte triggers membrane movement, but, because of the flat surface, phagocytosis does not occur, and lysosomal enzymes are released into the medium *(frustrated phagocytosis). Cytotoxic release* occurs after phagocytosis of potentially membranolytic substances, such as urate crystals. In addition, there is some evidence that certain granules, particularly the specific granules of neutrophils, may be directly secreted by *exocytosis.*[28]

Table 3–2. DEFECTS IN LEUKOCYTE FUNCTIONS

DISEASE	DEFECT
Genetic	
Leukocyte adhesion deficiency 1	Beta chain of CD11/CD18 integrins
Leukocyte adhesion deficiency 2	Sialylated oligosaccharide (receptor for selectin)
Neutrophil specific granule deficiency	Absence of neutrophil specific granules Defective chemotaxis
Chronic granulomatous disease	Decreased oxidative burst
X-linked	NADPH oxidase (membrane component)
Autosomal recessive	NADPH oxidase (cytoplasmic component)
Myeloperoxidase deficiency	Absent MPO-H_2O_2 system
Chédiak-Higashi syndrome	Multiple defects
Acquired	
Thermal injury, diabetes, malignancy, sepsis, immunodeficiencies	Chemotaxis
Hemodialysis, diabetes mellitus	Adhesion
Leukemia, anemia, sepsis, diabetes, neonates, malnutrition	Phagocytosis and microbicidal activity

Modified from Gallin, J.I. (ed): Disorders of phagocytic cells. *In* Inflammation: Basic Principles and Clinical Correlates, 2nd ed. New York, Raven Press, 1992, pp. 860 and 861.

Defects in Leukocyte Function

From the preceding discussion it is obvious that leukocytes play a cardinal role in host defense. Not surprisingly, therefore, defects in leukocyte function, both genetic and acquired, lead to increased vulnerability to infections (Table 3–2).[26] Impairments of virtually every phase of leukocyte function—from adherence to vascular endothelium to microbicidal activity—have been identified. We have described, for example, the genetic deficiencies in leukocyte adhesion molecules (LAD types 1 and 2) and in NADPH oxidase (CGD). Another is the *Chédiak-Higashi syndrome*, an autosomal recessive disorder characterized by neutropenia, defective degranulation, and delayed microbial killing. The neutrophils and other leukocytes have *giant granules*, resulting from fusion of predominantly azurophilic granules, which can be readily appreciated in peripheral blood smears. The molecular basis of this disease is unknown, but defects in microtubule function are suspected. Although individually rare, these genetic disorders underscore the importance of the complex series of leukocyte events that must occur *in vivo* following invasion by microorganisms.

SUMMARY OF THE ACUTE INFLAMMATORY RESPONSE

At this point it would be profitable to review the events in acute inflammation discussed so far. The vascular phenomena are characterized by increased blood flow to the injured area, resulting mainly from arteriolar dilatation and opening of capillary beds. Increased vascular permeability results in the accumulation of protein-rich extravascular fluid, which forms the exudate. Plasma proteins leave the vessels, most commonly through widened interendothelial cell junctions of the venules or by direct endothelial cell injury. The leukocytes, initially predominantly neutrophils, adhere to the endothelium via adhesion molecules, transmigrate across the endothelium, and migrate to the site of injury under the influence of chemotactic agents. Phagocytosis of the offending agent follows, which may lead to the death of the microorganism. During chemotaxis and phagocytosis, activated leukocytes may release toxic metabolites and proteases extracellularly, potentially causing tissue damage.

CHEMICAL MEDIATORS OF INFLAMMATION

Having described the events in acute inflammation, we can now turn to a discussion of the chemical mediators that account for the events. So many mediators have been identified that we are confronted with an embarrassment of riches. While the multitude may have survival value for the organism (and also for investigators searching for mediators and pharmaceutical companies for new drugs), they are most difficult for students to remember. Here we review general principles and highlight some of the more important mediators.

- *Mediators originate either from plasma or from cells* (Fig. 3–14). Plasma-derived mediators (e.g., complement) are present in plasma in *precursor forms* that *must be activated*, usually by a series of proteolytic cleavages, to acquire their biologic properties. Cell-derived mediators are normally *sequestered in intracellular granules* (e.g., histamine in mast cell granules) that need to be secreted or *are synthesized de novo* (e.g., prostaglandins) in response to a stimulus. The major cellular sources are platelets, neutrophils, monocytes/macrophages, and mast cells.
- Most mediators perform their biologic activity by initially *binding to specific receptors on target cells*. Some, however, have direct enzymatic activity (e.g., lysosomal proteases) or mediate oxidative damage (e.g., oxygen metabolites).
- *A chemical mediator can stimulate the release of mediators by target cells themselves.* These sec-

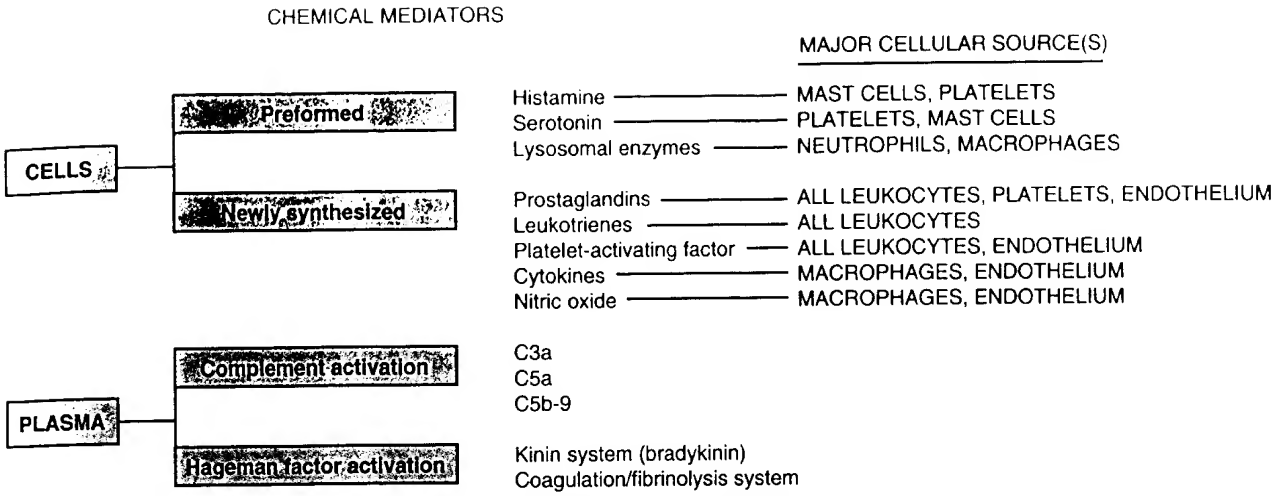


Figure 3–14. Chemical mediators of inflammation.

ondary mediators may be identical or similar to the initial mediators but may also have opposing activities. They provide mechanisms for amplifying—or in certain instances counteracting—the initial mediator action.

- Mediators can act on one or few target cell types, have widespread targets, or may even have differing effects, depending on cell and tissue types.
- *Once activated and released from the cell, most of these mediators are short-lived.* They quickly decay (e.g., arachidonic acid metabolites) or are inactivated by enzymes (e.g., kininase inactivates bradykinin), or they are otherwise scavenged (antioxidants scavenge toxic oxygen metabolites) or inhibited (e.g., complement inhibitors). There is thus a system of checks and balances in the regulation of mediator actions.
- *Most mediators may have harmful effects.* We shall now discuss the specific mediators.

VASOACTIVE AMINES

HISTAMINE. *Histamine* is widely distributed in tissues, the richest source being the mast cells that are normally present in the connective tissue adjacent to blood vessels (Fig. 3–15). It is also found in blood basophils and platelets. Preformed histamine is present in mast cell granules and is released by mast cell degranulation in response to a variety of stimuli: (1) physical injury such as trauma, cold, or heat; (2) immune reactions involving binding of antibodies to mast cells (see Chapter 6); (3) fragments of complement called anaphylatoxins (C3a and C5a); (4) histamine-releasing proteins derived from leukocytes; (5) neuropeptides (e.g., substance P); and (6) cytokines (IL-1, IL-8).

In humans, histamine causes dilatation of the arterioles and increases vascular permeability of the venules (it, however, *constricts* large arteries). It is considered to be the principal mediator of the immediate phase of increased vascular permeability, causing venular endothelial contraction and widening of the interendothelial cell junctions, as we have seen. It acts on the microcirculation mainly via H_1 receptors.

SEROTONIN. *Serotonin* (5-hydroxytryptamine) is a second preformed vasoactive mediator with actions similar to those of histamine. It is present in platelets and enterochromaffin cells, and in mast cells in rodents but not humans.

Release of serotonin (and histamine) from *platelets* is stimulated when platelets aggregate after contact with collagen, thrombin, adenosine diphosphate (ADP), and antigen-antibody complexes. Platelet aggregation and release are also stimulated by platelet-activating factor (PAF) de-

Figure 3–15. A flat spread of omentum showing mast cells *(dark blue)* around blood vessels and in the interstitial tissue. Stained with metachromatic stain to identify the mast cell granules. The red structures are fat globules stained with fat stain. (Courtesy of Dr. G. Majno.)

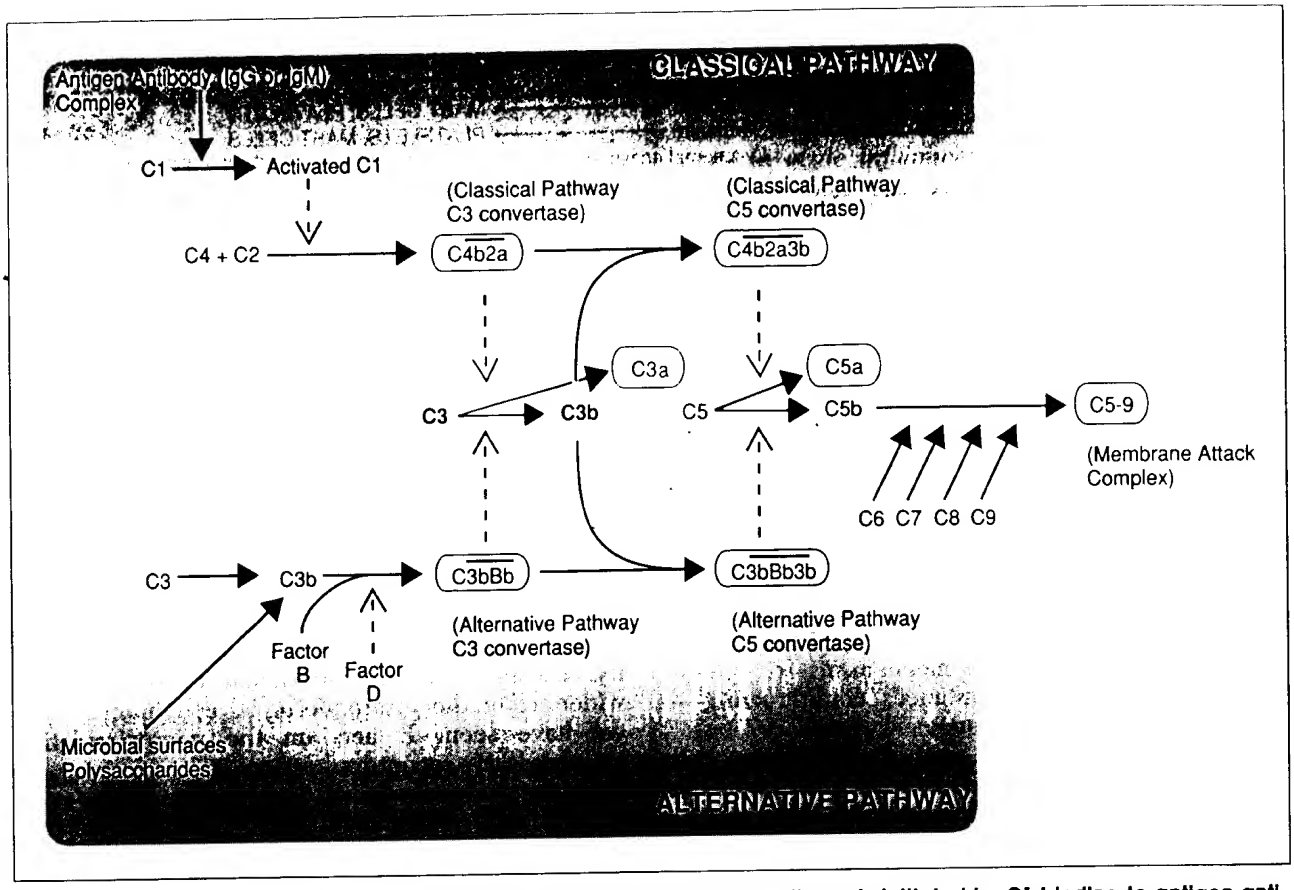


Figure 3–16. Overview of complement activation pathways. The classic pathway is initiated by C1 binding to antigen-antibody complexes, and the alternative pathway is initiated by C3b binding to various activating surfaces, such as microbial cell walls. The C3b involved in alternative pathway initiation may be generated in several ways, including spontaneously, by the classic pathway, or by the alternative pathway itself (see text). Both pathways converge and lead to the formation of inflammatory complement mediators (C3a and C5a) and the membrane attack complex. In this figure, bars over the letter designations of complement components indicate enzymatically active forms, and dashed lines indicate proteolytic activities of various components. (Modified from Abbas, A.K., et al.: Cellular and Molecular Immunology, 2nd ed. Philadelphia, W.B. Saunders Co., 1994.)

rived from mast cells during IgE-mediated reactions. In this way the platelet release reaction results in increased permeability during immunologic reactions. As will be discussed later, PAF itself has many inflammatory properties.

PLASMA PROTEASES

A variety of phenomena in the inflammatory response are mediated by three inter-related plasma-derived factors: the complement, kinin, and clotting systems (Figs. 3–16 and 3–17).

The Complement System

The complement system consists of 20 component proteins (together with their cleavage products), which are found in greatest concentration in plasma. This system functions in immunity for defense against microbial agents, culminating in lysing microbes by the so-called membrane attack complex (MAC). In the process, a number of complement components are elaborated that cause increased vascular permeability, chemotaxis, and opsonization (see Fig. 3–16).

Complement components present as inactive forms in plasma are numbered C1 through C9. Although it is not our intention to go into the detailed sequence of the activation of the "complement cascade," a brief review of the salient features will be helpful. The most critical step in the elaboration of the biologic functions of complement is the activation of the third component, C3. Cleavage of C3 can occur by the so-called *classical pathway,* which is triggered by fixation of C1 to antibody (IgM or IgG) combined with antigen, or through the *alternative pathway,* which can be triggered by microbial surfaces (e.g., endotoxins), aggregated IgA, complex polysaccharides, endotoxins, cobra venom, and so forth. The alternative pathway involves the participation of a distinct set of serum components called the properdin system (properdin [P], factors B and D). Whichever pathway is involved, *C3 convertase* splits C3 into two critical fragments—C3a, which is released, and C3b. C3b then binds to the fragments to form

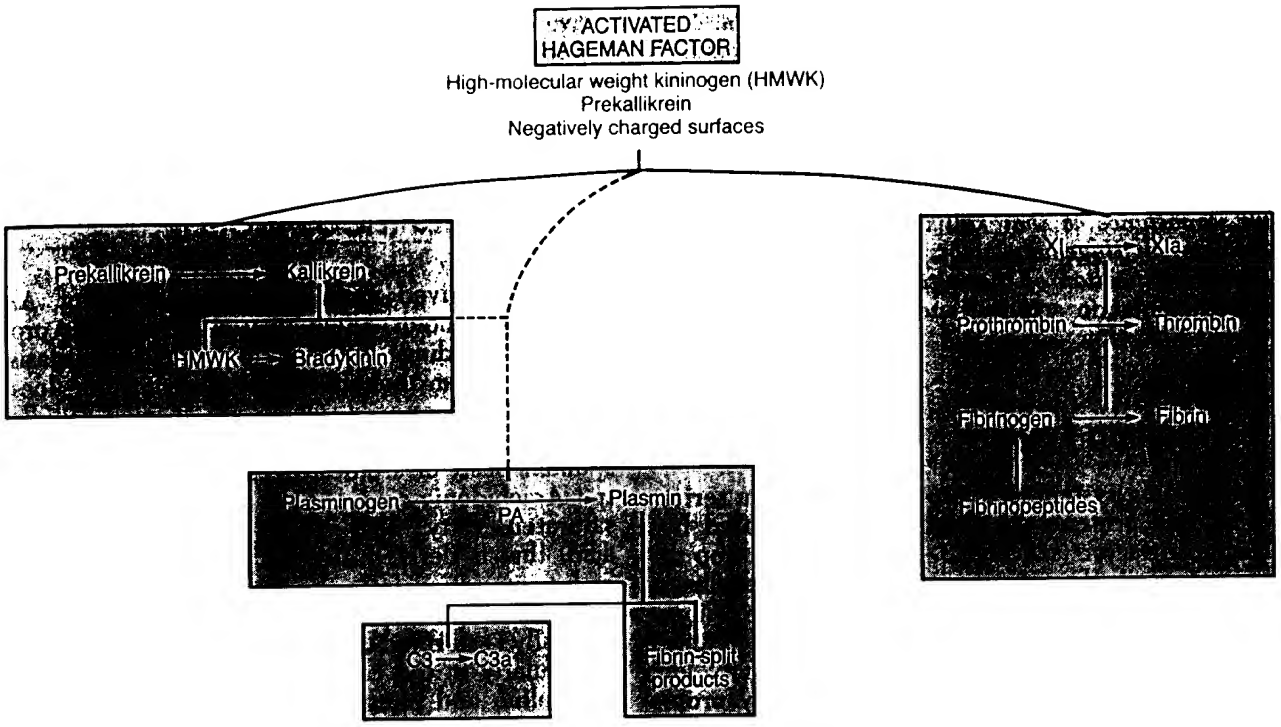


Figure 3–17. Inter-relations between the four plasma mediator systems. Interrupted lines indicate that the pathway may not be physiologically important. PA = plasminogen activator.

C5 convertase, which interacts with C5 to release C5a and initiate the assembly of the membrane attack complex (C5-C9). MAC causes lysis by initial hydrophobic binding to the lipid bilayer of target cells, eventually forming transmembrane channels.

Complement derived factors affect a variety of phenomena in acute inflammation:

- *Vascular phenomena. C3a, C5a,* and, to a small extent, *C4a* (called anaphylatoxins) are the split products of the corresponding complement components (see Fig. 3–16). They increase vascular permeability and cause vasodilatation mainly by releasing histamine from mast cells. C5a also activates the lipoxygenase pathway of arachidonic acid (AA) metabolism in neutrophils and monocytes, causing further release of inflammatory mediators.
- *Leukocyte adhesion, chemotaxis, and activation. C5a* is a powerful chemotactic agent for neutrophils, monocytes, eosinophils, and basophils. It also increases the adhesion of leukocytes to endothelium by activating the leukocytes and increasing the avidity of surface integrins to their endothelial ligand. C5a is rapidly converted in human serum to *C5a des Arg,* which is also chemotactic in the presence of a serum polypeptide called *cochemotaxin.*
- *Phagocytosis.* C3b and $C3b_i$, when fixed to the bacterial cell wall, act as an opsonin and favor phagocytosis by neutrophils and macrophages, which bear cell surface receptors for C3b.

Among the complement components, C3 and C5 are the most important inflammatory mediators. Their significance is further enhanced by the fact that, in addition to the mechanisms discussed above, C3 and C5 can be activated by several proteolytic enzymes present within the inflammatory exudate. These include plasmin and lysosomal enzymes released from neutrophils (see discussion later in this chapter). Thus, the chemotactic effect of complement and the complement-activating effects of neutrophils can set up a self-perpetuating cycle of neutrophil emigration.

The Kinin System

The kinin system is directly triggered by contact (surface) activation of Hageman factor (factor XII of the intrinsic clotting pathway) described below and in Chapter 4 (see Fig. 3–17). The kinin system results in the ultimate release of the vasoactive nonapeptide *bradykinin,* a potent agent that increases vascular permeability. *Bradykinin also causes contraction of smooth muscle, dilation of blood vessels, and pain when injected into the skin.* The cascade that eventually produces kinin is shown in Figure 3–17. It is triggered, as noted, by activation of Hageman factor by contact with negatively charged surfaces, such as collagen and basement membranes. A fragment of factor XII (prekallikrein activator, or factor XIIa) is produced, and this converts plasma prekallikrein into an

active proteolytic form, the enzyme *kallikrein*. The latter cleaves a plasma-glycoprotein precursor, *high-molecular-weight kininogen (HMWK)*, to produce *bradykinin* (HMWK also acts as a cofactor or catalyst in the activation of Hageman factor). The action of bradykinin is short-lived because it is quickly inactivated by an enzyme called *kininase*. *Of importance is that kallikrein itself is a potent activator of Hageman factor, allowing for autocatalytic amplification of the initial stimulus.* Kallikrein has chemotactic activity, and it also directly converts C5 to C5a.

The Clotting System

The clotting system (see also Chapter 4) is a series of plasma proteins that can also be activated by Hageman factor (see Fig. 3–17). The final step of the cascade is the conversion of fibrinogen to fibrin by the action of thrombin. During this conversion, *fibrinopeptides* are formed, which induce increased vascular permeability and chemotactic activity for leukocytes. *Thrombin* also has inflammatory properties, including causing increased leukocyte adhesion and fibroblast proliferation (see Chapter 4, Fig. 4–4).

The *fibrinolytic system* contributes to the vascular phenomena of inflammation in several ways, (Fig. 3–17). Plasminogen activator (released from endothelium, leukocytes, and other tissues) cleaves plasminogen, a plasma protein that binds to the evolving fibrin clot to generate *plasmin*, a multifunctional protease. (Kallikrein and activated Hageman factor can also convert plasminogen to plasmin *in vitro*, but the physiologic relevance of this is uncertain.) Plasmin is important in lysing fibrin clots, but in the context of inflammation it also cleaves C3 to produce C3 fragments, and it degrades fibrin to form "fibrin split products," which may have permeability-inducing properties.

From this discussion of the complex mediators generated by the kinin, complement, and clotting systems, a few general conclusions can be drawn:

- *Bradykinin, C3a, and C5a (as mediators of increased vascular permeability), and C5a (as the mediator of chemotaxis),* and *thrombin* (which has effects on many cell types) are the most likely to be important *in vivo*.
- *C3 and C5* can be generated by three different groups of influences: (a) classic immunologic reactions; (b) alternative complement pathway activation; and (c) agents with little immunologic specificity, such as bacterial products, plasmin, kallikrein, and some serine proteases found in normal tissue.
- *Activated Hageman factor (factor XIIa)* initiates the clotting, kinin and possibly the fibrinolytic systems. Some of the products of this initiation —particularly kallikrein—can, by feedback, activate Hageman factor, resulting in profound amplification of the effects of the initial contact.

ARACHIDONIC ACID (AA) METABOLITES: PROSTAGLANDINS AND LEUKOTRIENES (EICOSANOIDS)

Products derived from the metabolism of AA (called eicosanoids) affect a variety of biologic processes, including inflammation and hemostasis. They are best thought of as *autocoids*, or local short-range hormones, which are formed rapidly, exert their effects locally, and then either decay spontaneously or are destroyed enzymatically.

Arachidonic acid (AA) is a 20-carbon polyunsaturated fatty acid (5,8,11,14-eicosatetraenoic acid) that is derived directly from dietary sources or by conversion from the essential fatty acid *linoleic acid*. It does not occur free in the cell but is normally esterified in membrane phospholipids, particularly in the carbon 2 position of phosphatidylcholine and phosphatidylinositol. It is released from phospholipids through the activation of cellular phospholipases by mechanical, chemical, and physical stimuli or by other mediators (e.g., C5a). AA metabolism proceeds along one of two major pathways (Fig. 3–18) named after the enzymes that initiate the reactions.

- The *cyclooxgenase pathway* leads to the generation of *prostaglandins*. These include PGE_2, PGD_2, $PGF_{2\alpha}$, PGI_2 (prostacyclin), and thromboxane (TxA_2), each of which is derived by the action of a specific enzyme. Some of these enzymes have restricted tissue distribution. For example, platelets contain the enzyme thromboxane synthetase, and hence TxA_2 is the major product in these cells. TxA_2, a potent platelet-aggregating agent and vasoconstrictor, is itself unstable and rapidly converted to its inactive form TxB_2. Vascular endothelium, on the other hand, lacks thromboxane synthetase but posseses prostacyclin synthetase, which leads to the formation of prostacyclin (PGI_2) and its stable end product $PGF_{1\alpha}$. Prostacyclin is a vasodilator and a potent inhibitor of platelet aggregation. The opposing roles of TxA_2 and PGI_2 in hemostasis are further discussed in Chapter 4. PGD_2 is the major metabolite of the cyclooxygenase pathway in mast cells; along with PGE_2 and PGF_2 (which are more widely distributed) it causes vasodilatation and potentiates edema formation. Aspirin and nonsteroidal anti-inflammatory agents, such as indomethacin, inhibit cyclooxygenase and thus inhibit prostaglandin synthesis. Lipoxygenase, however, is not affected by these anti-inflammatory agents.
- In the *lipoxygenase pathway*, 5-lipoxygenase is the predominant enzyme in neutrophils, and the

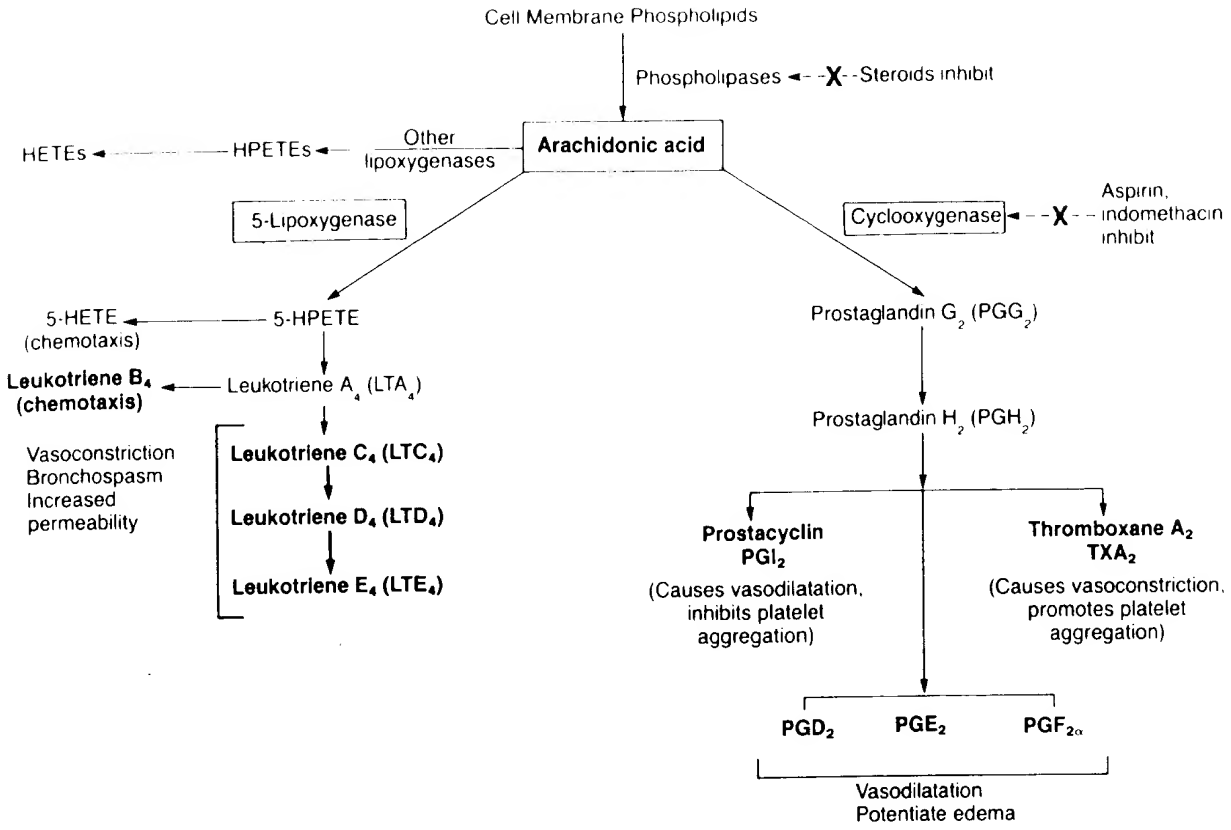


Figure 3–18. Generation of arachidonic acid metabolites and their roles in inflammation.

metabolites derived by its actions are the best characterized. The main product, 5-HETE, which is chemotactic for neutrophils, is converted into a family of compounds collectively called *leukotrienes*. LTB_4 is a potent chemotactic agent and causes aggregation of neutrophils. LTC_4, LTD_4, and LTE_4 cause vasoconstriction, bronchospasm, and increased vascular permeability. Neutrophils also produce trihydroxymetabolites of AA called *lipoxins*.[32] Lipoxins have both anti- and proinflammatory effects, and their role *in vivo* is currently being pursued.

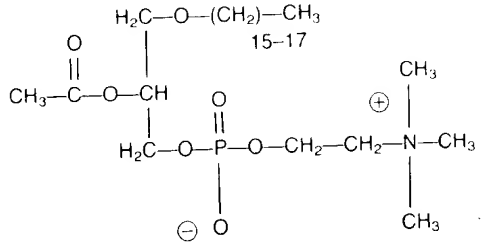
Eicosanoids can mediate virtually every step of acute inflammation (Table 3–3).

- *Prostaglandin E and prostacyclin* are important mediators of inflammatory vasodilation. They also markedly potentiate the permeability-increasing and chemotactic effects of other mediators.
- *The cysteinyl-containing leukotrienes C_4, D_4, and E_4* cause intense vasoconstriction and increase vascular permeability. The vascular leakage, as with histamine, is restricted to venules. They are also potent bronchoconstrictors.
- *Leukotriene B_4* causes aggregation and adhesion of leukocytes to venular endothelium and is a powerful chemotactic agent. Some of the other products of lipoxygenase metabolism, such as HETE, are also chemotactic.
- *The prostaglandins* are also involved in the pathogenesis of *fever* and *pain* in inflammation. PGE_2 causes a marked increase in pain produced by intradermal injection of suboptimal concentrations of histamine and bradykinin and interacts with cytokines in causing fever during infections, as described later in this chapter.
- Eicosanoids can be found in inflammatory exudates, and agents that suppress cyclooxygenase (aspirin, indomethacin) also suppress inflammation *in vivo*. Glucocorticoids, which are powerful anti-inflammatory agents, may act at least in part by inducing the synthesis of a protein that inhibits phospholipase A_2.
- Finally, variations in AA metabolism may account for some of the beneficial effects of *fish oil*. Diets rich in fish oil contain essential fatty acids of the

Table 3–3. INFLAMMATORY ACTIONS OF EICOSANOIDS

ACTION	METABOLITE
Vasoconstriction	Thromboxane A_2, leukotrienes C_4, D_4, E_4
Vasodilatation	PGI_2, PGE_1, PGE_2, PGD_2
Increased vascular permeability	Leukotrienes C_4, D_4, E_4
Chemotaxis	Leukotriene B_4, HETE

SOURCES	MAJOR INFLAMMATORY ACTIONS
Mast cells/basophils	Increased vascular permeability
Neutrophils	Leukocyte aggregation
Monocytes/macrophages	Leukocyte adhesion
Endothelium	Leukocyte priming/ chemotaxis
Platelets	Platelet activation
Others	Stimulation of other mediators (LT, $O_2^{\cdot}$)



PLATELET ACTIVATING FACTOR

Figure 3–19. Structure, sources, and main inflammatory actions of PAF. LT = leukotrienes.

ω 3 variety (e.g., *linolenic acid*) rather than ω 2 linoleic acid found in most animal or vegetable fat. The ω 3 fatty acids serve as poor substrates for conversion to active metabolites of the cyclooxygenase and, particularly, the lipoxygenase series. Such diets inhibit platelet aggregation and thrombosis and prevent certain inflammatory processes.

PLATELET-ACTIVATING FACTOR

Platelet-activating factor (PAF) is another phospholipid-derived mediator.[34] Its name comes from its initial discovery as a factor derived from antigen-stimulated IgE-sensitized basophils, which causes platelet aggregation and release but is now known to have multiple inflammatory effects. Chemically, it is an acetyl glycerol ether phosphocholine and it is synthesized from membrane phospholipids by activation of phospholipases (PLA_2) (Fig. 3–19). In addition to platelet stimulation, PAF causes vasoconstriction and bronchoconstriction, and at extremely low concentrations it induces vasodilation and increased venular permeability with a potency 100 to 10,000 times greater than that of histamine. PAF also causes increased leukocyte adhesion to endothelium (by enhancing leukocyte integrin binding), chemotaxis, degranulation, and the oxidative burst. Thus, PAF can elicit most of the cardinal features of inflammation. A variety of cell types, including basophils, neutrophils, monocytes, and endothelial cells, can elaborate PAF, in both secreted and cell-bound forms.

PAF acts directly on target cells via specific receptors, but it also boosts the synthesis of other mediators, particularly eicosanoids, by leukocytes and other cells. A role for PAF *in vivo* is supported by the ability of synthetic PAF antagonists to inhibit inflammation in some experimental models.

CYTOKINES

Cytokines are polypeptides produced by many cell types (but principally activated lymphocytes and macrophages) that modulate the function of other cell types. Long known to be involved in cellular immune responses (see Chapter 6), these products have additional effects that play important roles in the inflammatory response.[35] The main cytokines that mediate inflammation are IL-1, TNF (α and β), and the IL-8 family.

IL-1 and *TNF* share many biologic properties. IL-1 and TNF-α are produced by activated macrophages, TNF-β by activated T cells, and IL-1 by many other cell types as well. Their secretion can be stimulated by endotoxin, immune complexes, toxins, physical injury, and a variety of inflammatory processes. Like growth factors, they induce their effects in three ways: they can act on the same cell that produces them (an *autocrine* effect), on cells in the immediate vicinity (as in lymph nodes and joint spaces; a *paracrine* effect); or systemically, as with any other hormone (*endocrine* effect). Their most important actions in inflammation are their effects on endothelium, leukocytes, and fibroblasts, and induction of the systemic acute-phase reactions (Fig. 3–20). The latter are relevant to the process of repair and are described in Chapter 2 and later in this chapter. In endothe-

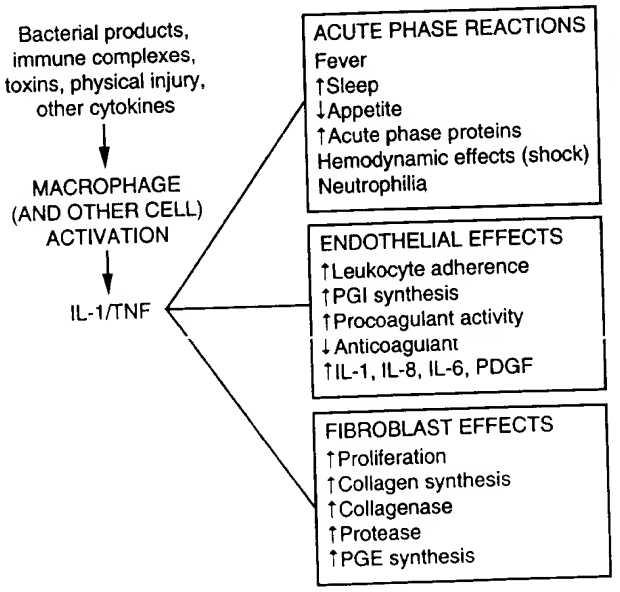


Figure 3–20. Major effects of interleukin-1 (IL-1) and tumor necrosis factor (TNF) in inflammation.

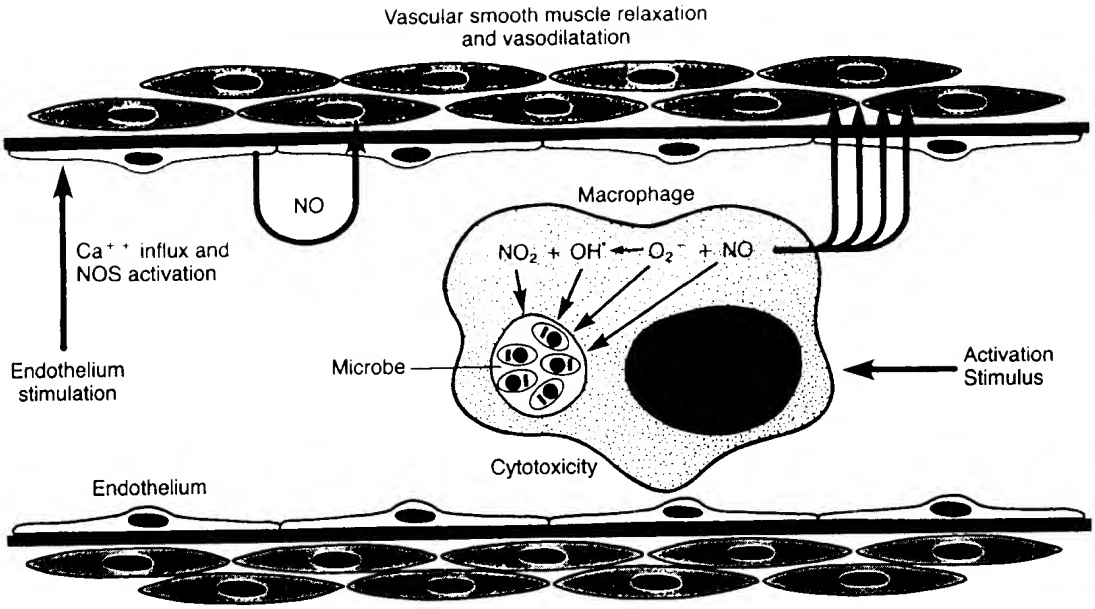


Figure 3–21. Two types of NO synthesis in endothelium *(left)* and macrophages *(right)*. NO causes vasodilation and NO free radicals are cytotoxic to microbial and mammalian cells. NOS = nitric oxide synthase. (Courtesy of Dr. Jeffrey Joseph.)

lium, they induce a spectrum of changes—mostly regulated at the level of gene transcription—referred to as *endothelial activation.*[13] In particular, they induce the synthesis of endothelial adhesion molecules, other cytokines, growth factors, eicosanoids, and nitric oxide and increase surface thrombogenicity of the endothelium. TNF also causes aggregation and *priming* of neutrophils, leading to augmented responses of these cells to other mediators, and the release of proteolytic enzymes from mesenchymal cells, thus contributing to tissue damage.

IL-8 is a small (8000 MW) polypeptide secreted by activated macrophages and other cell types (e.g., endothelial cells) that is a powerful chemoattractant and activator of neutrophils, with limited activity on monocytes and eosinophils.[36] Its most important inducers are other cytokines, mainly IL-1 and TNF-α. It belongs to a family of structurally similar small proteins, now called *chemokines,*[36] characterized by four cysteine residues at identical positions. These include, in addition to IL-8, *platelet factor 4 (PF4),* a cationic protein of platelet alpha granules (see Chapter 4) with chemotactic activity for neutrophils, monocytes, and eosinophils and with histamine-releasing activity for mast cells; *monocyte chemoattractant protein (MCP-1),* a chemotactic and activating agent that is fairly specific for monocytes, and *RANTES,* which is chemotactic for thymocytes. The chemokines bind to a family of receptors, called *serpentines,* characterized by seven transmembrane domains coupled to G proteins.[37]

Both IL-1 and TNF (as well as IL-6) also induce the systemic *acute-phase responses* (see discussion under section on fever)[38] associated with infection or injury, including fever, the production of slow-wave sleep, the release of neutrophils into the circulation, the release of adrenocorticotropic hormone (ACTH) and corticosteroids, and, particularly with regard to TNF, the hemodynamic effects of septic shock—hypotension, decreased vascular resistance, increased heart rate, and decreased blood pH.

NITRIC OXIDE (NO)

This is a relatively "new" mediator of inflammation, and the history of its discovery deserves brief mention. In 1980, Furchgott showed that vasodilatation produced by acetylcholine requires an intact endothelium.[39] In response to such vasodilatory agents, endothelial cells produced a short-acting factor, endothelium-derived relaxing factor (EDRF), that relaxed vascular smooth muscle. Subsequently, it was shown that vascular endothelium produces NO, and this gas has the physical and biologic properties of EDRF.[40] NO binds to the heme moiety on guanylyl cyclase and activates the enzyme. Through a cascade of kinases, the resultant increase in the concentration of cyclic guanosine monophosphate (cGMP) mediates relaxation and, consequently, produces vasodilatation.

Nitric oxide is a soluble free radical gas that is produced not only by endothelial cells but also by macrophages and specific neurons in the brain. *It is synthesized from L-arginine, molecular oxygen, and NADPH by the enzyme nitric oxide synthase (NOS).* There are two types of NOS (Fig. 3–21). In endothelial cells and neurons, *NOS* is present

A

SPECIFIC GRANULES
- Lactoferrin
- Lysozyme
- Alkaline phosphatase
- Type IV collagenase
- Leukocyte adhesion molecules
- Plasminogen activation
- Phospholipase A_2

AZUROPHIL GRANULES
- Myeloperoxidase
- Lysozyme–Bactericidal factors
- Cationic proteins
- Acid hydrolases
- Elastase
- Nonspecific collagenase
- BPI
- Defensins
- Cathepsin G
- Phospholipase A_2

B

Figure 3–22. Ultrastructure of neutrophil granules stained for peroxidase activity and their constituents. The large peroxidase-containing granules are the azurophil granules; the smaller peroxidase-negative ones are the specific granules (SG). N = portion of nucleus; BPI = bactericidal permeability increasing protein.

constitutively and can be activated rapidly by an increase in cytoplasmic calcium ions in the presence of calmodulin. Influx of calcium into these cells leads to a rapid production of NO. In contrast, *macrophage NOS is induced,* when macrophages are activated by cytokines (e.g., IFN-γ) or other agents; no intracellular increase in calcium is required. Since the *in vivo* half-life of NO is only a matter of seconds, the gas acts only on cells in close proximity to where it is produced. Such a localized activity accounts for the specificity of its actions.

In addition to vascular smooth muscle relaxation, NO plays other important roles in inflammation. It reduces platelet aggregation and adhesion, as described in Chapter 4. The NO produced by macrophages, acting as a free radical, is cytotoxic to certain microbes and tumor cells. It can oxidize sulfhydryl groups on proteins and cause a depletion of cytosolic glutathione, and it can react with superoxide anion to form the strong oxidant nitrogen dioxide and the highly reactive hydroxyl radical ($NO^{\bullet} + O_2^{-} \longrightarrow NO_2^{-} + OH^{\bullet}$).[40] Uncontrolled NO production by activated macrophages in septic shock (see Chapter 4) can lead to massive peripheral vasodilatation and shock, and NO has also been implicated in a variety of inflammatory diseases. Inhibitors of NO production are now being tested clinically and have been shown, for example, to reduce the size of ischemic brain infarcts.

LYSOSOMAL CONSTITUENTS OF LEUKOCYTES

Neutrophils and monocytes contain lysosomal granules, which when released may contribute to the inflammatory response. *Neutrophils* exhibit two main types of granules (Fig. 3–22). The smaller *specific* (or secondary) granules contain lactoferrin, lysozyme, alkaline phosphatase, the components of NADPH oxidase, the intracytoplasmic pool of integrins, and (in human neutrophils) a collagenase. The large *azurophil* (or primary) granules contain MPO, bactericidal factors (lysozyme, defensins), acid hydrolases, and a variety of neutral proteases (elastase, nonspecific collagenases, proteinase 3). As previously explained, these enzymes are either released after cell death or secreted by a variety of mechanisms. There are, however, differences in the mobilization of specific and azurophil granules. The specific granules are secreted more readily and by lower concentrations of agonists, while the more potentially destructive azurophil granules release their contents primarily within the phagosome and require very high levels of agonists to be released extracellularly.[42]

Acid proteases degrade proteins at an acid pH. Their most likely action is to degrade bacteria and debris *within* the phagolysosomes, where an acid pH is readily reached. *Neutral proteases,* on the other hand, are capable of degrading various extracellular components. These enzymes can attack collagen, basement membrane, fibrin, elastin, and cartilage, resulting in the tissue destruction characteristic of purulent and deforming inflammatory processes. Neutral proteases can also cleave C3 and C5 directly, releasing anaphylatoxins, and release a kinin-like peptide from kininogen. *Monocytes* and *macrophages* also contain acid hydrolases, collagenase, elastase, and plasminogen activator. These may be particularly active in chronic inflammatory reactions.

Lysosomal constituents thus have numerous effects. The initial leukocytic infiltration, if unchecked, can potentiate further increases in vascular permeability, chemotaxis, and tissue damage. These harmful proteases, however, are held in check by a system of *antiproteases* in the serum and tissue fluids. Foremost among these is *alpha$_1$-antitrypsin,* which is the major inhibitor of neutrophilic elastase. A

deficiency of these inhibitors may lead to sustained action of leukocyte proteases, as is the case in patients with alpha$_1$-antitrypsin deficiency (see Chapter 15). *Alpha$_2$-macroglobulin* is another antiprotease found in serum and various secretions.

OXYGEN-DERIVED FREE RADICALS

These metabolites may be released extracellularly from leukocytes after exposure to chemotactic agents, immune complexes, or a phagocytic challenge. Their production is dependent, as we have seen, on the activation of the NADPH oxidative system and generation of superoxide. Superoxide, in turn, is converted to H_2O_2, OH,$^{\bullet}$ and, by combining with NO, to toxic NO derivatives.[43] They are implicated in the following responses.

- *Endothelial cell damage, with resultant increased vascular permeability.* Adherent neutrophils, when activated, not only produce their own toxic species but also stimulate xanthine oxidation in endothelial cells themselves, thus elaborating more superoxide.
- *Inactivation of antiproteases,* such as alpha$_1$-antitrypsin, discussed earlier together with *activation of metalloproteinases.* This leads to unopposed protease activity, with increased destruction of ECM.
- *Injury to other cell types* (tumor cells, red cells, parenchymal cells).

Serum, tissue fluids, and target cells possess antioxidant protective mechanisms that detoxify these potentially harmful oxygen-derived radicals. These antioxidants have been discussed in Chapter 1, but to repeat, they include (1) the copper-containing serum protein *ceruloplasmin;* (2) the iron-free fraction of serum, *transferrin;* (3) the enzyme *superoxide dismutase,* which is found or can be activated in a variety of cell types; (4) the enzyme *catalase,* which detoxifies H_2O_2; and (5) *glutathione peroxidase,* another powerful H_2O_2 detoxifier. *Thus, the influence of oxygen-derived free radicals in any given inflammatory reaction depends on the balance between the production and the inactivation of these metabolites by cells and tissues.*

OTHER MEDIATORS

Neuropeptides, such as substance P, cause vasodilatation and increased vascular permeability both directly and by stimulating histamine release and ei-

Table 3–4. SUMMARY OF MEDIATORS OF ACUTE INFLAMMATION

MEDIATOR	SOURCE	ACTION		
		Vascular Leakage	*Chemotaxis*	*Other*
Histamine and serotonin	Mast cells, platelets	+	−	
Bradykinin	Plasma substrate	+	−	Pain
C3a	Plasma protein via liver; macrophages	+	−	Opsonic fragment (C3b)
C5a		+	+	Leukocyte adhesion, activation
Prostaglandins	Most cells, from membrane phospholipids	Potentiate other mediators	−	Vasodilation, pain, fever
Leukotriene B_4	Leukocytes	−	+	Leukocyte adhesion, activation
Leukotriene C_4, D_4, E_4	Leukocytes, mast cells	+	−	Bronchoconstriction, vasoconstriction
Oxygen metabolites	Leukocytes	+	±	Endothelial damage, tissue damage
PAF	Leukocytes; mast cells	+	+	Bronchoconstriction Leukocyte priming
IL-1 and TNF	Macrophages; other	−	+	Acute phase reactions Endothelial activation
IL-8	Macrophages Endothelium	−	+	Leukocyte activation
Nitric oxide	Macrophages Endothelium			Vasodilation Cytotoxicity

Table 3–5. MOST LIKELY MEDIATORS IN INFLAMMATION

VASODILATION
Prostaglandins
Nitric oxide
INCREASED VASCULAR PERMEABILITY
Vasoactive amines
C3a and C5a (through liberating amines)
Bradykinin
Leukotrienes C_4, D_4, E_4
PAF
CHEMOTAXIS, LEUKOCYTE ACTIVATION
C5a
Leukotriene B_4
Bacterial products
Cytokines (IL-8)
FEVER
IL-1, IL-6, TNF
Prostaglandins
PAIN
Prostaglandins
Bradykinin
TISSUE DAMAGE
Neutrophil and macrophage lysosomal enzymes
Oxygen metabolites
Nitric oxide

cosanoid production by mast cells, and also enhance neutrophil adhesion and chemotaxis.[44]

Growth factors (platelet-derived growth factor [PDGF] and transforming growth factor β [TGF-β]), as we described in Chapter 2, may be chemotactic to leukocytes and mesenchymal cells and have other activities resembling those of cytokines. Indeed, there is so much overlap in the functions of cytokines and growth factors that the terms are now used interchangeably. Additionally, certain *ECM components* or their fragments have chemotactic activity. These will be discussed further under the section on chronic inflammation.

SUMMARY OF CHEMICAL MEDIATORS OF ACUTE INFLAMMATION

Table 3–4 summarizes the major actions of the principal mediators. When Lewis suggested that existence of histamine, one mediator was clearly not enough. Now, we are wallowing in them! Yet, from this menu of substances we can tentatively extract a few mediators that may be relevant *in vivo* (Table 3–5). *For increased vascular permeability, histamine, the anaphylatoxins (C3a and C5a), the kinins, leukotrienes C, D, and E, and PAF are almost certainly involved, at least early in the course of inflammation. For chemotaxis, complement fragment C5a, lipoxygenase products (LTB_4), and other chemotactic lipids are the most likely protagonists. Also, the important role of prostaglandins in vasodilation, pain, and fever and in potentiating edema cannot be denied. IL-1 and TNF are involved with endothelial-leukocyte interactions and with acute-phase reactions. Lysosomal products and oxygen-derived radicals are the most likely candidates as causes of the ensuing tissue destruction. NO is involved in vasodilatation and cytotoxicity.*

OUTCOMES OF ACUTE INFLAMMATION

The discussion of mediators completed the basic description of the relatively uniform pattern of the inflammatory reaction encountered in most injuries. *Recall that, although hemodynamic, permeability, and white cell changes have been described sequentially and may be initiated in this order, all these phenomena in the fully evolved reaction to injury are concurrent in a seemingly chaotic but remarkably organized multiring circus. As might be expected, many variables may modify this basic process, including the nature and intensity of the injury, the site and tissue affected, and the responsiveness of the host.*

In general, however, acute inflammation may have one of four outcomes (Fig. 3–23):

1. *Complete resolution.* In a perfect world, all inflammatory reactions, once they have succeeded in neutralizing the injurious stimulus, should end with restoration of the site of acute inflammation to normal. This is called *resolution* and is the usual outcome when the injury is limited or short-lived or when there has been little tissue destruction. Resolution involves neutralization of the chemical mediators, with subsequent return of normal vascular permeability, cessation of leukocytic infiltration, and finally removal of edema fluid and protein, leukocytes, foreign agents, and necrotic debris from the battleground (Fig. 3–24). Lymphatics and phagocytes play a role in these events, as we shall see.

2. *Healing by connective tissue replacement (fibrosis)* occurs after substantial tissue destruction, or when the inflammatory injury occurs in tissues that do not regenerate, or when there is abundant fibrin exudation. When the fibrinous exudate in tissue or serous cavities (pleura, peritoneum) cannot be adequately resorbed, connective tissue grows into the area of exudate, converting it into a mass of fibrous tissue—a process also called *organization.*

3. *Abscess formation,* which occurs particularly in infections with pyogenic organisms.

4. Progression of the tissue response to *chronic inflammation,* which will be discussed next, may follow acute inflammation, or the response may be chronic almost from the onset. Acute to chronic transition occurs when the acute inflammatory response cannot be resolved, owing either to the persistence of the injurious agent or to some interference in the normal process of healing. For example, bacterial infection of the lung may begin as a focus of acute inflammation (pneumonia), but

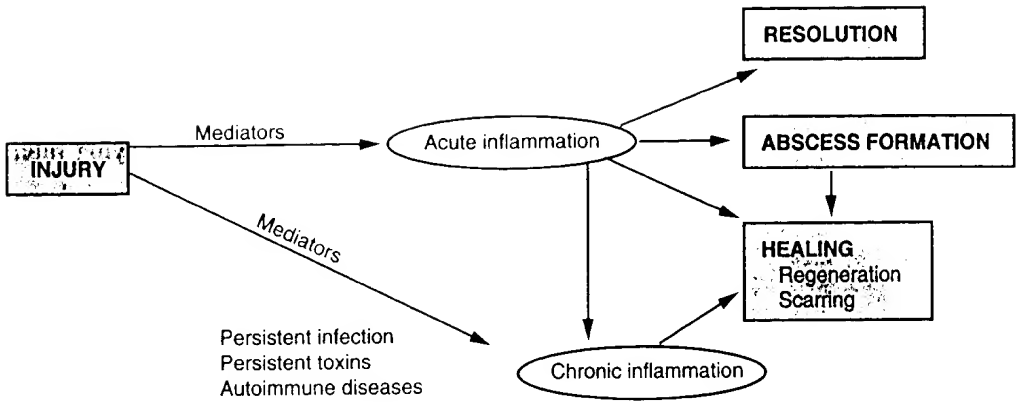


Figure 3–23. Outcome of acute inflammation (see text).

its failure to resolve may lead to extensive tissue destruction and formation of a cavity in which the inflammation continues to smolder, leading eventually to a chronic lung abscess. Another example of chronic inflammation with a persisting stimulus is peptic ulcer of the duodenum or stomach. Peptic ulcers may persist for months or years and, as we shall see, are manifested by both acute and chronic inflammatory reactions.

We shall now proceed to a more detailed account of chronic inflammation.

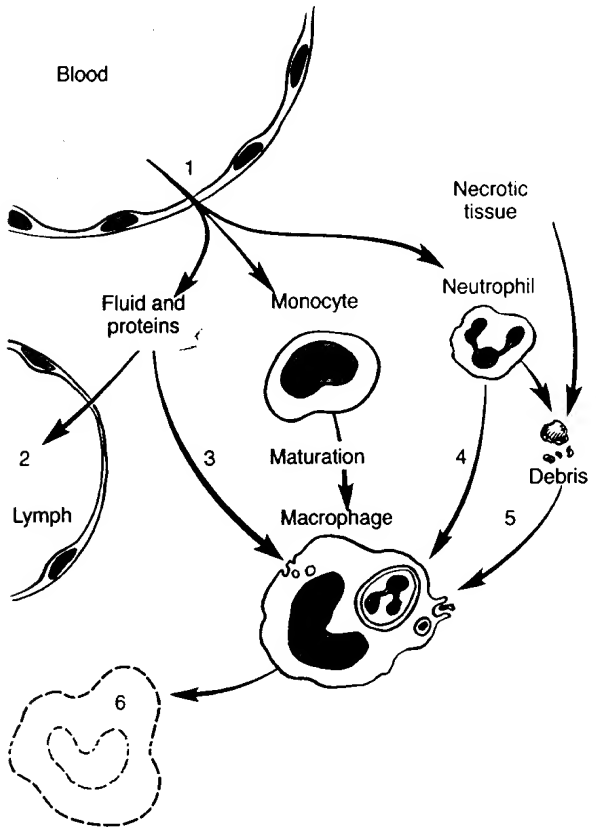


Figure 3–24. Events in the resolution of inflammation: (1) return to normal vascular permeability; (2) drainage of edema fluid and proteins into lymphatics or (3) by pinocytosis into macrophages; (4) phagocytosis of neutrophils; (5) necrotic debris by macrophages; and (6) disposal of macrophages. Note the central role of macrophages in resolution. (Modified from Haslett, C., and Henson, P.M.: *In* Clark, R., and Henson, P.M. (eds.): The Molecular and Cellular Biology of Wound Repair. New York, Plenum Press, 1988.)

CHRONIC INFLAMMATION

Although difficult to define precisely, chronic inflammation is considered to be *inflammation of prolonged duration* (weeks or months) *in which active inflammation, tissue destruction, and attempts at healing are proceeding simultaneously.* While it may follow acute inflammation, as described earlier, chronic inflammation frequently begins insidiously, as a low-grade, smoldering, often asymptomatic response. Indeed, this latter type of chronic inflammation includes some of the most common and disabling of human diseases, such as rheumatoid arthritis, atherosclerosis, tuberculosis, and the chronic lung diseases. Such inflammation arises under the following settings:

- *Persistent infections* by certain microorganisms, such as tubercle bacilli, *Treponema pallidum* (causative organism of syphilis), and certain fungi. These organisms are of low toxicity and evoke an immune reaction called delayed hypersensitivity (see Chapter 6). The inflammatory response sometimes takes a specific pattern called a *granulomatous reaction*, which is discussed later in this chapter.
- *Prolonged exposure to potentially toxic agents, either exogenous or endogenous.* Examples are nondegradable inanimate material, such as particulate silica inhaled for a prolonged period that results in a lung inflammatory disease called *silicosis* (Chapter 15); and plasma lipid components, which, if chronically elevated, induce *atherosclerosis* (Chapter 11).
- Under certain conditions, immune reactions are set up against the individual's own tissues, lead-

Figure 3–25. Chronic inflammation in the lung, showing all three characteristic histologic features: a collection of chronic inflammatory cells; replacement by connective tissue (fibrosis); and destruction of lung parenchyma—normal alveoli are replaced by spaces lined by cuboidal epithelium.

ing to *autoimmune diseases* (see Chapter 6). In these diseases, autoantigens evoke a self-perpetuating immune reaction that results in several common chronic inflammatory diseases, such as rheumatoid arthritis and lupus erythematosus.

In contrast to acute inflammation, which is manifested by vascular changes, edema, and largely neutrophilic infiltration, *chronic inflammation is characterized by (1) infiltration with mononuclear cells, which include macrophages, lymphocytes, and plasma cells,* a reflection of a persistent *reaction to injury,* (2) *tissue destruction,* largely induced by the inflammatory cells, and (3) attempts at *repair by connective tissue replacement,* namely proliferation of small blood vessels *(angiogenesis)* and, in particular, *fibrosis* (Fig. 3–25). We shall now review these manifestations of chronic inflammation and discuss the mechanisms underlying them.

MONONUCLEAR INFILTRATION

The *macrophage* is the prima donna of chronic inflammation, and we shall begin our discussion with a brief review of its biology.[46]

Macrophages are but one component of the *mononuclear phagocyte system (MPS),* previously known as the reticuloendothelial system (RES) (Fig. 3–26). The MPS consists of closely related cells of bone marrow origin, including blood monocytes, and tissue macrophages. The latter are diffusely scattered in the connective tissue or clustered in organs such as the liver (Kupffer's cells), spleen and lymph nodes (sinus histiocytes), and lungs (alveolar macrophages). All arise from a common precursor in the bone marrow, which gives rise to blood monocytes. From the blood, monocytes migrate into various tissue and transform into macrophages. The half-life of blood monocytes is about one day, whereas the life span of tissue macrophages is several months.

As discussed previously, monocytes begin to emigrate relatively early in acute inflammation, and within 48 hours they constitute the predominant cell type. Extravasation of monocytes is governed by the same factors involved in neutrophil emigration, namely adhesion molecules and chemical mediators with chemotactic and activating

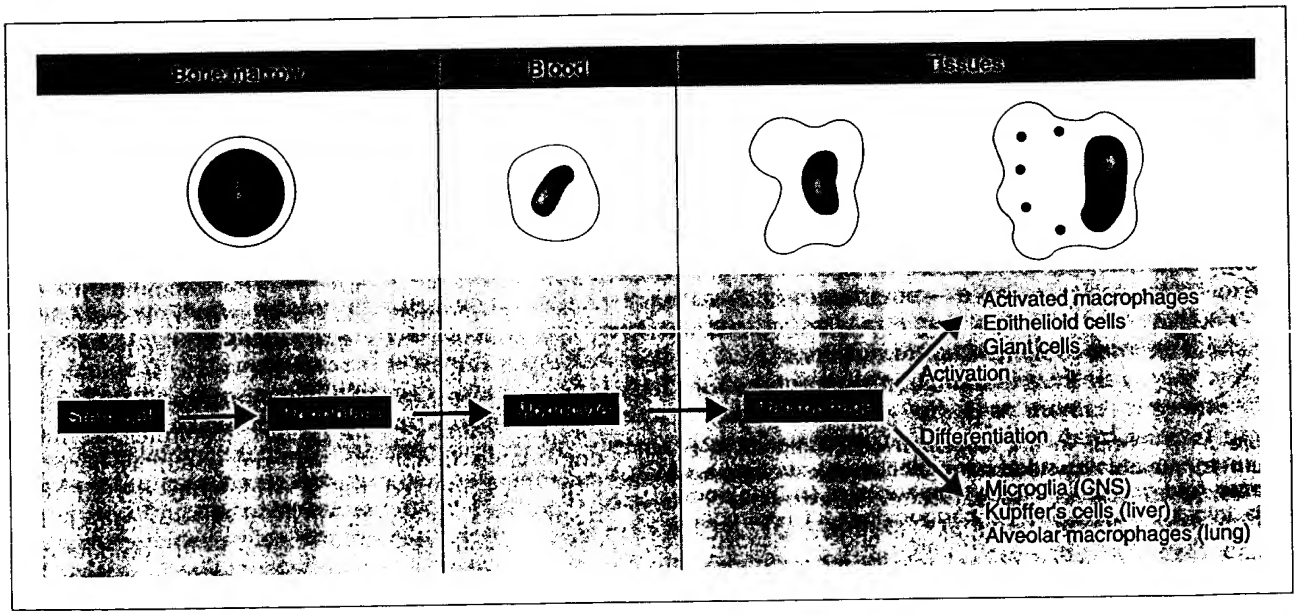


Figure 3–26. Maturation of mononuclear phagocytes. (With permission from Abbas, A. K., et al.: Cellular and Molecular Immunology, 2nd ed. Philadelphia, W.B. Saunders Co., 1994.)

EXHIBIT

I

Infectious Susceptibility and Severe Deficiency of Leukocyte Rolling and Recruitment in E-Selectin and P-Selectin Double Mutant Mice

By Daniel C. Bullard,* Eric J. Kunkel,‖ Hiroshi Kubo,¶ M. John Hicks,‡ Isabel Lorenzo,§ Nicholas A. Doyle,¶ Claire M. Doerschuk,¶ Klaus Ley,‖ and Arthur L. Beaudet*§

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Summary

During the initial phase of the inflammatory response, leukocytes marginate and roll along the endothelial surface, a process mediated largely by the selectins and their ligands. Mice with mutations in individual selectins show no spontaneous disease and have mild or negligible deficiencies of inflammatory responses. In contrast, we find that mice with null mutations in both endothelial selectins (P and E) develop a phenotype of leukocyte adhesion deficiency characterized by mucocutaneous infections, plasma cell proliferation, hypergammaglobulinemia, severe deficiencies of leukocyte rolling in cremaster venules with or without addition of TNF-α, and an absence of neutrophil emigration at 4 h in response to intraperitoneal *Streptococcus pneumoniae* peritonitis. These mice provide strong evidence for the functional importance of selectins in vivo.

Leukocyte and endothelial cell adhesion molecules play an important role in inflammatory and immune responses (1, 2). The initial steps in leukocyte emigration in response to inflammatory stimuli involve leukocyte rolling that is mediated primarily by interactions between selectins and selectin ligand molecules (3). P-selectin is expressed on endothelium and platelets, E-selectin on endothelium, and L-selectin on the majority of leukocytes. Selectins bind to carbohydrate portions of glycoproteins that serve as selectin ligands, many of which are mucin-like proteins (4–7). Leukocyte rolling is followed by firm attachment and emigration, processes largely dependent on the interaction of the β_2 leukocyte integrins and immunoglobulin family members including iCAM-1 and related molecules (1, 8). The leukocyte integrins are heterodimeric proteins and include LFA-1 ($\alpha_L\beta_2$), Mac-1 ($\alpha_M\beta_2$), p150,95 ($\alpha_X\beta_2$), and $\alpha_d\beta_2$.

The functional importance of these adhesion molecules is demonstrated by the occurrence of two human genetic disorders, leukocyte adhesion deficiency types I and II (LAD I/II)[1] (9). LAD I is caused by genetic deficiency of the common β-subunit of the β_2 leukocyte integrins (CD18) and is characterized by increased granulocyte counts, impaired leukocyte emigration, and susceptibility to life-threatening bacterial infections (10, 11). LAD II is caused by an unspecified defect in fucose metabolism and is also characterized by increased granulocyte counts, and recurrent bacterial infections (12, 13). The defect in fucose metabolism is thought to affect the carbohydrate portion of selectin ligand molecules. The known LAD II patients also suffer from a variety of disorders outside the immune system leading to severe mental retardation and short stature (12). The functional abnormality in LAD I is primarily impairment of firm adhesion and emigration, whereas impaired leukocyte rolling is characteristic of LAD II (9, 14, 15).

We are studying the functional importance and the effects of genetic deficiency for the selectin molecules. The three known selectins (L, P, and E) occur as a gene cluster in mice and humans (16), and null mutations for each of the individual genes have been reported using gene targeting in mice (17–20). Modest reductions of leukocyte rolling and emigration occur in mice lacking L-selectin or P-selectin, but not in mice lacking E-selectin, and all three mutant strains remain healthy under normal laboratory conditions (17–21). To further define the role of selectins in inflam-

[1] *Abbreviations used in this paper:* ES, embryonic stem; LAD, leukocyte adhesion deficiency.

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matory responses, we have prepared a line of mice with null mutations in both endothelial selectins (P and E); and find that these mice demonstrate spontaneous susceptibility to infections with a phenotype of leukocyte adhesion deficiency.

Materials and Methods

Gene Targeting for E-Selectin. A cDNA for mouse E-selectin (gift of Christie Ballantyne, Baylor College of Medicine) was used to screen a 129/Sv genomic library (Stratagene, La Jolla, CA); genomic clones were partially characterized using restriction digests and PCR and compared to the published genomic structure (22). An E-selectin replacement construct was generated by replacing ~1.4 kb of DNA between the PstI site in exon 4 and the HindIII site in exon 6 with an HPRT minigene (23); (gift of Allan Bradley, Baylor College of Medicine). This construct was electroporated into P-selectin mutant AB2.1 ES cells (24) carrying the previously described P-selectin mutation (20). Digestion with EcoRI and hybridization with the probe indicated were used to identify homologous recombinants on Southern blots Several clones were isolated that correctly targeted the E-selectin gene, and injection of one of these clones into C57BL/6 embryos resulted in germline transmission. This construct was also used to generate mice deficient in E-selectin alone following electroporation into unmanipulated AB2.1 embryonic stem (ES) cells. All mice used in these experiments were from a mixed 129/Sv and C57BL/6 background and were kept in a specific pathogen-free barrier facility. Only mutant mice that had no hair loss and no symptoms of opportunistic infection or lymphadenopathy were used in the experiments described.

Pathology. Necropsies were performed on 12 affected E-/P-selectin-deficient mice and compared with four age-matched control mice. Tissue was obtained from major organ systems as well as cervical and perioral skin, gingiva, and oral mucosa. The tissue was fixed in formalin, embedded in paraffin and examined following hematoxylin and eosin staining. Additional tissue was cryopreserved at −80°C for immunocytochemical studies. Bacterial and fungal cultures were performed from lymph node, lung, liver, spleen, and blood. Methenamine silver and gram stains, for the detection of fungi and bacteria, were performed on tissue sections. Immunocytochemical studies were performed on deparaffinized sections of the enlarged cervical lymph nodes using kappa and lambda light chain antibodies (Dakopatt, Carpintera, CA) to determine if the plasmacytoid lymphocytic proliferation was monoclonal. Portions of lung were fixed in glutaraldehyde and processed for electron microscopic examination.

Intravital Microscopy Experiments. Mice were anesthetized exactly as described (21). Some animals were pretreated with an intrascrotal injection of 0.5 μg mouse TNF-α (Genzyme, Cambridge, MA) in 0.3 ml saline for 2 to 2.5 h. The cremaster muscle was prepared for intravital microscopy as described (21, 25), and superfused with thermocontrolled (37°C) bicarbonate-buffered saline as described (26). Microscopic observations were made on a Zeiss Axioscope (salt water immersion objective 40/0.75 numerical aperture) as described previously (27). Microvascular centerline red blood cell velocity was measured using a dual phototransistor and an automatic tracking correlator implemented on a personal computer based on software described previously (28). Throughout the experiment, small blood samples (20 μl each) were withdrawn from the carotid catheter at ~45-min intervals and analyzed for leukocyte concentration (hemocytometer) and differential leukocyte counts (Leukostat, Fisher-Scientific, Pittsburgh, PA). Microvessel diameter was measured interactively using a digital image processing system (28), and rolling leukocyte flux was determined by counting the number of leukocytes passing each venule as described (21). Total leukocyte flux was estimated as the product of measured systemic leukocyte concentration and blood volume flow calculated from the venule cross-sectional area multiplied with mean blood flow velocity. Leukocyte rolling flux fraction is defined as the flux of rolling leukocytes as a percentage of total leukocyte flux, which is independent of variations in systemic leukocyte counts. Leukocyte rolling flux fraction in E-selectin/P-selectin-deficient mice was compared with age-matched wild-type controls using Student's *t*-test.

Peritonitis Studies. Anesthetized mice received an intravenous injection of ^{125}I-albumin into the tail vein, and edema formation was calculated as described (20). After 15 min, *Streptococcus pneumoniae* (1–5 × 10^9 organisms/mouse) were injected in the peritoneal space. After either 4 or 24 h (n = 5 in each group), the mice inhaled an overdose of halothane. The peritoneal space was lavaged using 5 ml of phosphate-buffered saline three times for a total of 15 ml. The circulating neutrophil counts and neutrophil counts in the peritoneal lavage fluid were quantitated using a hemocytometer and Wright-stained smears on cytospin preparations. The clearance of bacteria from the peritoneal space was determined by measuring the number of colony-forming units (CFU) in the peritoneal instillate and in the peritoneal lavage fluid after 4 or 24 h.

Results

Gene Targeting of E- and P-Selectin. Since E-selectin and P-selectin are tightly linked members of a gene cluster (16), the double mutation could not be obtained by breeding the single mutations, and it was necessary to introduce both mutations onto a single chromosome in ES cells. An ES cell line carrying the previously reported P-selectin mutation (20) was electroporated with a plasmid construct designed to produce a null mutation in E-selectin (Fig. 1 *a*). Only 50% of the targeted cell clones would be expected to carry both mutations on the same chromosome as is required to produce double homozygous mice. Chimeric males obtained from one clone, P2D6, transmitted both mutations on the same chromosome to their progeny, and double homozygous mice were obtained by intercrossing as documented by Southern blotting (Fig. 1 *b*). The same plasmid construct was used to obtain mice carrying the null mutation for E-selectin alone.

Phenotype of E-/P-Selectin Deficient Mice. There was no evidence for prenatal or early postnatal lethality in the double homozygous mice. The evidence that the P selectin mutation represents a null allele was published previously (20). The E-selectin mutation is predicted to be a null mutation since a portion of exon 4, all of exon 5, and a portion of exon 6 are deleted, leaving no mechanism for synthesis of the normal protein (Fig. 1 *a*). Immunohistochemical staining of lung tissue from the E-/P-selectin double mutant mice, and from the single mutant mice using monoclonal antibodies against E- or P-selectin, failed to show any reactivity for the mutated gene(s) after intratracheal administration of *Escherichia coli* endotoxin (data not shown).

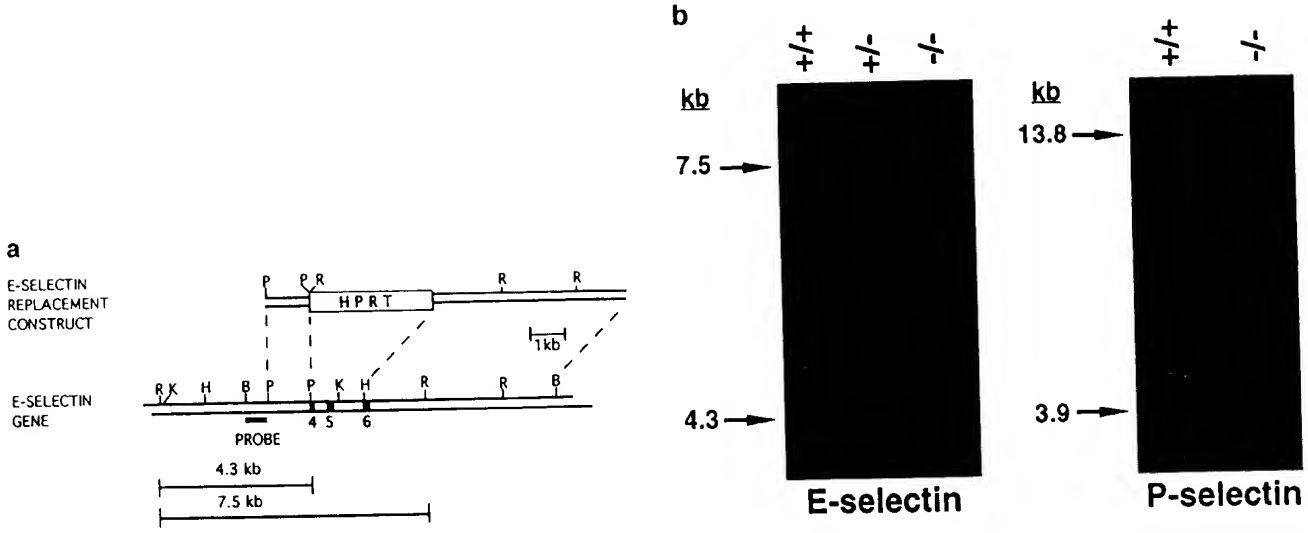


Figure 1. Gene targeted mutations for E-selectin and P-selectin. (*a*) E-selectin replacement construct and partial restriction map of the endogenous E-selectin locus. Exons 4, 5, and 6 are indicated as solid boxes; the positions of the other exons are not shown. *P*, PstI; *R*, EcoRI; *B*, BamHI; *H*, HindIII; not all PstI sites are shown. The HindIII site present in exon 6 and the BamHI site present at the 3′ end of the construct were destroyed in the construct. The E-selectin-specific probe indicated in *a* and a probe specific for the P-selectin gene (20), were hybridized separately to tail DNA from nonmutant, double heterozygote, and double homozygote mutant mice. The E-selectin probe identifies two EcoRI fragments of 4.3 and 7.5 kb corresponding to the mutated and endogenous E-selectin locus, respectively. The P-selectin probe identifies a 3.9-kb endogenous EcoRV fragment and a 13.8-kb EcoRV mutant fragment. (*b*) blot confirmation of E- and P-selectin mutations.

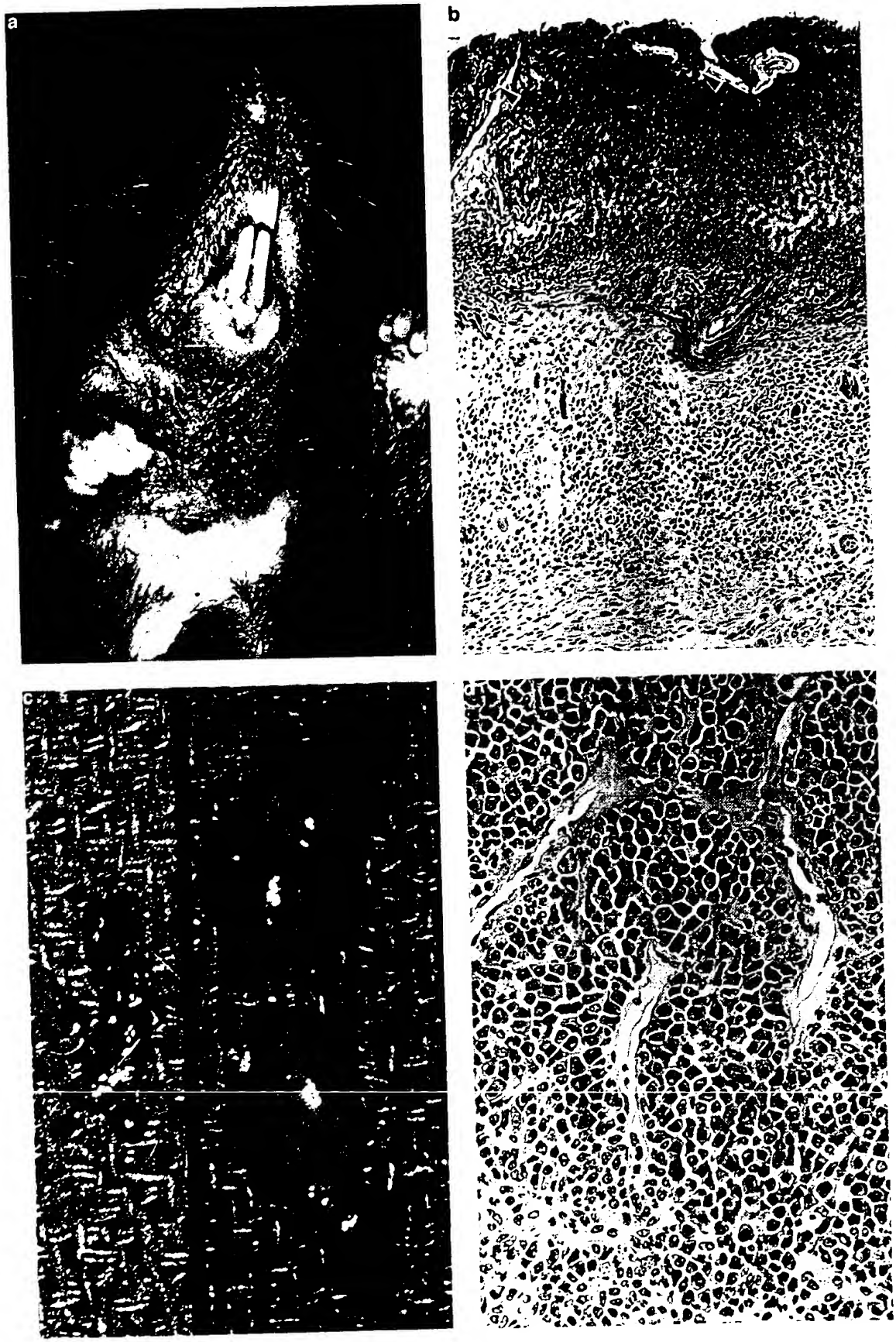
In contrast to the healthy status of mice carrying either the E- or P-selectin mutation alone, double homozygous mice began to show abnormalities at variable ages after weaning. Findings included nonulcerative excoriative skin lesions with hair loss in the head and neck regions, generalized redness and swelling of the oral mucosa, and overgrowth of the mandibular and maxillary incisors (Fig. 2 *a*). Clipping of the incisors regularly was necessary for the mice to feed. These manifestations were present in a few mice by the time of weaning, and the proportion of affected mice increased continuously so that >90% had obvious signs of the disease by 6–8 mo of age.

Leukocyte counts in E-/P-selectin double mutant mice without obvious signs of disease were extremely elevated for neutrophils, lymphocytes, and monocytes; mean/μl ± SEM were as follows: 12,020 ± 200 neutrophils for mutants versus 1690 ± 690 for controls; 10,930 ± 1224 lymphocytes for mutants versus 4930 ± 249 for controls; and 873 ± 188 monocytes for mutants versus 148 ± 13 for controls (all $P < 0.001$). Blood smears revealed a marked increase in immature as well as mature neutrophils.

Histologic sections from affected skin and oral mucosa showed loss of the overlying squamous epithelium and superficial colonization of these tissue surfaces by bacteria (Fig. 2 *b*); cultures were positive for *Staphylococcus aureus*, *Escherichia coli*, *Enterococcus faecium*, and other common bacterial flora. These organisms did not invade the underlying dermis or submucosa, and these regions contained a mixed granulocytic and mononuclear cell infiltrate. Histologic sections of the oral cavity also demonstrated periodontitis with a chronic leukocytic infiltrate of the periodontal tissue and evidence of gingival recession. A few mice spontaneously developed conjunctivitis with bacterial flora similar to that recovered from infected skin. Repetitive cultures for bacterial and fungal organisms taken from blood, spleen, liver, lymph node, and lung were negative.

Necropsy examination also revealed massive cervical lymphadenopathy (Fig. 2 *c*) due to replacement of the nodal architecture with an infiltrate consisting predominantly of plasmacytoid lymphocytes and plasma cells on histologic examination (Fig. 2 *d*). Increased numbers of plasma cells were also observed in sections from spleen and bone marrow in affected mice. Immunohistochemical staining of plasma cells with kappa or lambda chain antibodies was positive for both, indicating that the proliferation was not monoclonal (data not shown). The IgG levels in serum of double mutant mice were increased ~10-fold; mean ± SEM were as follows: 34,760 ± 4312 μg/ml for mutants versus 3339 ± 708 μg/ml for controls ($P < 0.0001$). IgM levels were not significantly increased. The accumulation of plasma cells in the cervical lymph nodes is unusual compared to what is observed in chronic bacterial infections due to various forms of leukocyte dysfunction and may imply a defect in plasma cell traffic in addition to the chronic infection (9).

Paraffin-embedded histologic sections of lung tissue revealed an increased cellularity in the alveolocapillary walls and no emigration of leukocytes into the alveolar space or the airways. The most striking observation on ultrastructural studies was that the increased cellularity was due to a large increase in the number of neutrophils and other leukocytes marginated within the capillary lumina. Many of


a
b

these marginated neutrophils (24%) showed ultrastructural features of apoptosis. Almost no neutrophils had spontaneously migrated into either the interstitium or the alveolar spaces.

Leukocyte Rolling. In order to investigate the effects of the combined deficiency of E- and P-selectin on leukocyte rolling, we used intravital microscopy to determine the leukocyte rolling flux fraction in venules of the cremaster muscle (21, 27). In this model, the muscle was exteriorized (which provides a mild inflammatory stimulus), and rolling was assessed for 2 h. In P-selectin-deficient mice, rolling is initially absent, and the rolling leukocyte flux fraction increases over time (21). In contrast, L-selectin mutant mice display normal initial rolling with the fraction of rolling leukocytes declining significantly at later times (21). We have not observed a significant reduction in rolling flux fraction in E-selectin mutant mice (data not shown). However, leukocyte rolling is induced in P-selectin mutant or P-selectin/ICAM-1 double mutant mice by TNF-α, a cytokine that stimulates expression of both E- and P-selectin, and this rolling is blocked by antibodies to either E- or L-selectin (21, 27). In the E-/P-selectin double mutant mice studied before onset of obvious disease symptoms, leukocyte rolling was completely absent in venules of the cremaster muscle at all time points up to 2 h after exteriorization and when mice were pretreated with TNF-α (Fig. 3). The leukocyte rolling flux was <0.5% of leukocytes passing through the vessels in all cases. Isolated leukocytes were seen to firmly attach to the vessel wall in TNF-α treated mice.

Streptococcus Pneumoniae-induced Peritonitis We also examined neutrophil emigration during *Streptococcus pneumoniae*-induced peritonitis to further assess leukocyte function in double mutant mice. Previous studies in mice with single selectin deficiencies have shown a positive correlation between impairment of leukocyte rolling in P-selectin- and L-selectin-deficient mice with reductions in neutrophil emigration during peritonitis (17, 19, 20). In response to *S. pneumoniae*, the E-/P-selectin double mutant mice showed complete absence of neutrophil emigration at 4 h after instillation compared to a moderate reduction in P-selectin-deficient mice and normal emigration in E-selectin-deficient mice (Table 1). Neutrophil emigration in double mutant mice was increased between 4 and 24 h, resulting in accumulation of similar numbers of intraperitoneal neutrophils compared to wild-type mice at 24 h. Edema formation in the peritonitis model correlated well with neutrophil emigration with significant reductions in double mutant mice and P-selectin-deficient mice at 4 h. Edema was significantly increased in E-/P-selectin double mutants at 24 h. Clearance of *S. pneumoniae* from the peritoneal space was not different for any of the mutant mice at 4 h, but was significantly reduced at 24 h in the double mutant mice.

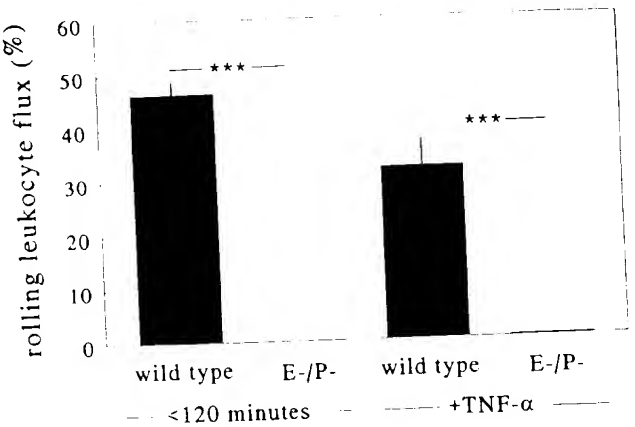


Figure 3. Leukocyte rolling flux fraction in untreated and TNF-α-treated venules in wild-type and E-selectin/P-selectin-deficient mice. Average leukocyte rolling flux fraction in untreated E-/P-selectin-deficient mice (82 venules) was significantly lower ($P < 0.001$) than in untreated wild-type mice (130 venules) 10–120 min after cremaster exteriorization. TNF-α-treated mice were pretreated with an intrascrotal injection 2–2.5 h before the initiation of surgery. Consistent with previous findings (21) TNF-α treatment does not increase the flux of rolling leukocytes in wild-type mice, primarily because firm adhesion is greatly increased in the TNF-α-treated venules. Rolling in TNF-α-treated E-/P-selectin-deficient mice (42 venules) is also significantly lower ($P < 0.01$) than that seen in similarly treated wild-type mice (17 venules). Data shown as mean ± SEM.

L-Selectin Expression. The defect observed in the E-/P-selectin double mutant mice is the most severe deficiency of leukocyte rolling described in any selectin-deficient mouse to date, suggesting that L-selectin and possible selectin-independent mechanisms are insufficient to provide the adhesive forces necessary for rolling. This observation is of particular interest in view of the deficiency of leukocyte rolling observed in L-selectin-deficient mice (19, 21), suggesting that L-selectin is required for effective rolling but by itself is not sufficient. To further clarify the role of L-selectin in E-/P-selectin mutant mice, its expression on the surface of peripheral blood leukocytes was examined by flow cytometry. Both older (8–10 wk) and younger (3 wk) mice displayed a substantial reduction (1/6 to 1/8 of normal mean channel fluorescence) of L-selectin expression on Gr-1-positive granulocytes (data not shown). Expression of L-selectin on Thy 1.2-positive lymphocytes was also reduced to about one half of normal. However, when CD45-positive bone marrow cells from E-/P-selectin double mutant mice were analyzed, the expression of L-selectin was 80–85% of normal. These observations suggest that the reduction in L-selectin on the surface of peripheral leukocytes may be due to increased shedding and activation in association with chronic infection (29, 30). Decreased levels of L-selectin expression on neutrophils from LAD I pa-

Figure 2. Gross morphology and histologic analysis of E-/P-selectin deficient mice. (*a*) Ventral view of the neck of an affected E-/P-selectin mutant with excoriative skin lesions and gingival recession. (*b*) Histopathologic appearance of affected skin with bacterial colonization (*arrows*) of the epidermis and a mixed inflammatory dermal infiltrate. (*c*) Markedly enlarged cervical lymph nodes from an affected double mutant (*right*) compared with normal (*left*). (*d*) Histopathologic appearance of a cervical lymph node from an affected animal.

Table 1. *S. pneumoniae-induced Peritonitis*

Mutation	4 h			24 h		
	Neutrophil accumulation	Edema formation	Bacteria recovered	Neutrophil accumulation	Edema formation	Bacteria recovered
Wild type	0.57 ± 0.15	8.0 ± 0.8	40 ± 7	4.55 ± 2.04	3.2 ± 0.5	0.3 ± 0.3
P-selectin	0.19 ± 0.003*	3.8 ± 1.1*	44 ± 10	2.84 ± 0.39	3.9 ± 0.6	16 ± 14
E-selectin	0.37 ± 0.07	5.7 ± 1.5	32 ± 14	2.56 ± 0.18	2.9 ± 0.5	28 ± 28
E-/P-selectin	0.06 ± 0.01*	2.1 ± 0.7*	39 ± 13	6.51 ± 1.76	6.9 ± 1.1‡	102 ± 53‡
Wild type, no organisms	0.01 ± 0.01	0.5 ± 0.1	N.D.	0.01 ± 0.00	0.9 ± 0.1	N.D.
E-/P-selectin, no organisms	N.D.	N.D.	N.D.	0.04 ± 0.01	1.3 ± 0.1	N.D.

Neutrophil emigration is expressed as the number of neutrophils × 10^{-5}/ml peritoneal lavage fluid. The formation of edema is expressed as the percent of ^{125}I-albumin recovered in the peritoneal fluid. Clearance of *S. pneumoniae* from the peritoneal space is expressed as percent recovery of organisms instilled in the peritoneal lavage fluid. Data are expressed as mean ± SEM. N.D. = not determined.
*Significantly less than value observed in wild-type mice that received organisms, $P < 0.05$.
‡Significantly greater than value observed in wild-type mice that received organisms, $P < 0.05$.

tients, but normal expression on neutrophils from LAD II patients have been reported (15, 31). The absence of leukocyte rolling in the venules and of neutrophil emigration into the peritoneum of E/P-selectin double mutant mice may therefore result from complete loss of E- and P-selectin on endothelial cells and perhaps a partial loss of L-selectin on leukocytes.

Discussion

The role of selectins has been evaluated using one or more blocking monoclonal antibodies in wild-type mice and using single selectin mutations with or without additional monoclonal antibodies. The results indicate that inactivation of multiple selectins leads to severe defects in early leukocyte rolling and/or peritoneal emigration of neutrophils (18, 21, 32). Monoclonal antibodies are not suitable for chronic administration, but long term observations of single mutant mice reveals no evidence of spontaneous infection. Here we demonstrate genetic deficiency of E- and P-selectin in mice results in increased susceptibility to spontaneous infections over time. In the human syndrome LAD type II, a disease that affects the expression of sialyl Lewis X and thus selectin ligands, the two patients described suffer from recurrent bacterial infections, neutrophilia, and a decrease in neutrophil rolling (9, 12–15). Given the hypothesis that LAD type II is primarily a defect of selectin ligand expression on leukocytes, it is instructive to compare this with the double deficiency of endothelial selectins in mice. Generally the phenotyes are quite similar, although expression of L-selectin is normal on LAD II granulocytes and is reduced in the selectin-deficient mice. We have observed no evidence for neurological damage or abnormal growth patterns in the double mutant mice.

In the peritonitis studies reported here, the most remarkable finding was that the E-/P-selectin double mutant mice showed a complete defect in neutrophil emigration at 4 h, but no defect compared to wild-type mice at 24 h. This is in contrast to either single mutation, since the P-selectin mutant mice showed only a partial reduction at 4 h and no defect at 24 h, while E-selectin mutants were similar to wild-type mice at both time points. Thus, the initial deficiency of rolling in E-/P-selectin double mutant mice is not associated with absence of *S. pneumoniae*–induced neutrophil emigration at later times points. Leukocyte emigration is also not completely deficient in other tissues as illustrated by the observation of a mixed inflammatory infiltrate in the dermis and epidermis of infected skin from E-/P-selectin double mutant animals. This result suggests that other E- and P-selectin-independent mechanisms can mediate adhesion and emigration. It is possible that the poorly controlled bacterial proliferation provides a greater stimulus which now recruits additional adhesion pathways. There is other evidence for differences in adhesion molecules involved in acute inflammatory responses within the first 6 h compared to 24–48 h (33, 34). These double mutant mice will be extremely valuable for delineation of the mechanisms and molecules that allow neutrophil emigration at later times.

It is interesting that L-selectin mutant mice show partial reductions in neutrophil emigration into the peritoneal cavity at 4, 24, and 48 h during thioglycollate-induced peritonitis (19, 34). The reduction observed at later timepoints in L-selectin-deficient mice compared to normal emigration for the other mutants may indicate a separate role for L-selectin that is not shared by E- and P-selectin. However, the differences in the two models (a single chemical stimulus vs. proliferating bacteria) may affect the nature and the extent of the stimulation of adhesive pathways and may contribute to the differences observed at 24 h.

These data raise questions regarding the extent to which the functions of selectins (particularly E and P) are distinct

versus overlapping. The mutant mice described here demonstrate that the combined deficiency of E- and P-selectin causes a disease phenotype that is not seen with either deficiency alone. Given the evolutionary conservation of each of the selectins and the considerable differences in patterns of expression, we believe that the roles of E- and P-selectin are only partially overlapping. Preparation of mice with other combinations of mutations in selectins and selectin ligands and more detailed analysis of each mutant should further clarify the distinct roles of individual molecules.

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EXHIBIT

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Characterization of E-Selectin-Deficient Mice: Demonstration of Overlapping Function of the Endothelial Selectins

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Summary

The initial rolling interaction of leukocytes with the blood vessel wall during leukocyte trafficking has been postulated to rely on members of the selectin family of adhesion molecules. Two selectins, E-selectin and P-selectin, have been identified that are expressed on activated endothelial cells. Mice deficient in E-selectin expression have been produced in order to examine the role of this selectin in leukocyte trafficking. Mice homozygous for an E-selectin null mutation were viable and exhibited no obvious developmental alterations. E-selectin-deficient mice displayed no significant change in the trafficking of neutrophils in several models of inflammation. However, blocking both endothelial selectins by treatment of the E-selectin-deficient animals with an anti-murine P-selectin antibody, 5H1, significantly inhibited neutrophil emigration in two distinct models of inflammation. While neutrophil accumulation at early times during thioglycollate-induced peritonitis was dependent on P-selectin, neutrophil accumulation at later time points was blocked by 5H1 only in E-selectin-deficient mice but not in wild-type mice. Similarly, edema as well as leukocyte accumulation in a model of delayed-type hypersensitivity in the skin was almost completely prevented by blockade of P-selectin function with 5H1 in the E-selectin-deficient mice while the same treatment had no effect in wild-type mice. These data demonstrate that the majority of neutrophil migration in both models requires an endothelial selectin but that E-selectin and P-selectin are functionally redundant. These data have important implications in the use of selectin antagonists in the treatment of inflammatory disease.

Introduction

The ability of leukocytes to leave the circulation and enter specific tissues is critical for the local control of infections and for the repair of injured tissue. In addition, the inappropriate or abnormal accumulation of immune cells at specific sites is a central component in the development of a variety of autoimmune diseases and immune cell–mediated tissue injury. The process by which leukocytes migrate out of the circulation has been suggested to involve at least three distinct steps (Butcher, 1991; Springer, 1994). The initial interaction appears to be transient and of low affinity, resulting in the rolling of leukocytes along the blood vessel wall. Then, the leukocytes become activated, presumably owing to locally acting factors produced by the endothelium, resulting in the arrest and firm adhesion of cells to the endothelium. Finally, the leukocyte traverses the endothelial layer (diapedesis). The initial rolling of cells has been suggested to rely on the functions of the selectin family of proteins, while firm adhesion and diapadesis appear to be mediated by the interaction of immunoglobulin superfamily members expressed on endothelial cells with integrin molecules expressed on leukocytes (Von Andrian et al., 1991; Lawrence and Springer, 1991, 1993).

The selectin family of proteins consists of three members, L-selectin, P-selectin, and E-selectin (Bevilacqua et al., 1989; Johnston et al., 1989; Lasky et al., 1989; for review see Bevilacqua, 1993). All selectins share a highly conserved structure with an N-terminal lectin-like (LEC) domain, an epidermal growth factor–like (EGF) domain, a variable number of complement regulatory protein-like (CRs) domain, and a single transmembrane and short cytoplasmic domains. All selectins recognize ligands containing specific carbohydrate moieties (for review see Paulson, 1992), and structural studies have identified critical regions and amino acids within the LEC domain required for mediating this interaction (Kansas et al., 1991; Erbe et al., 1992; Graves et al., 1993). The ligands or coreceptors for the selectins all appear to be specific proteins or glycolipids, which are decorated with sialyl-Lewis X or highly related carbohydrates (Larson et al., 1990; Lasky et al., 1992; Walcheck et al., 1993; Baumhueter et al., 1993; Bochner et al., 1994). L-selectin (also known as Mel-14, lymphocyte homing receptor, or CD62L) was initially identified as a homing receptor for peripheral lymph nodes (Gallatin et al., 1983; Lasky et al., 1989) and is constitutively expressed on most leukocytes (Lewinsohn et al., 1987; Camerini et al., 1989; Griffin et al., 1990). P-selectin (also known as GMP-140, PADGEM, or CD62P) is present both in platelets and in endothelial cells (Larson et al., 1989; Geng et al., 1990). E-selectin (also known as ELAM-1 and CD62E) expression has been found only on endothelial cells (Bevilacqua et al., 1989). The three selectin molecules appear to be specialized for mediating the initial interaction of leukocytes with the endothelium under

conditions of flow found in the blood stream. Recombinant P-selectin and E-selectin can mediate rolling of leukocytes on an artificial surface in vitro under shear forces similar to those encountered by cells in the circulation (Lawrence and Springer, 1991, 1993). Also, anti-L-selectin antibodies or immunoglobulin G (IgG) L-selectin chimeric proteins can inhibit the rolling of endogenous leukocytes on rat mesenteric venules (Ley et al., 1991; Von Andrian et al., 1991). In contrast, the interaction of ICAM-1 with its counter receptor, LFA-1, on leukocytes can not mediate adhesion under conditions of flow. These observations suggest that the selectins mediate the initial interaction of leukocytes with endothelial cells and slow the leukocyte as a prerequisite to stable adhesion and extravasation mediated by integrins.

A large number of observations support a critical role for the selectins in inflammation. Blockade of L-selectin function by targeted gene disruption, antibody, or IgG L-selectin chimeric proteins partially inhibits neutrophil migration at early times but not late times in models of thioglycollate (TG)-induced peritonitis (Jutila et al., 1989; Watson et al., 1991; Arbones et al., 1994). P-selectin, which mediates the adhesion of a number of leukocytes (including neutrophils, monocytes, and eosinophils) to the endothelium, is prestored in Weibel–Palade bodies of endothelial cells, and is rapidly mobilized to the cell surface upon induction with a variety of inflammatory mediators, including histamine, thrombin, or calcium ionophores (McEver et al., 1989). Recent experiments indicate that, in some species, P-selectin transcription is also induced by cytokines and lipopolysaccharide (LPS) (Weller et al., 1992; Gotsch et al., 1994). Several experiments have demonstrated a role for P-selectin in vivo. Inflammation in the lungs of rats induced by cobra venom factor or IgA immune complexes can be inhibited by treatment with antibody to P-selectin (Mulligan et al., 1991, 1992). A role for P-selectin in neutrophil migration has recently been demonstrated using P-selectin-deficient mice (Mayadas et al., 1993). Mice deficient in P-selectin expression are viable and have no developmental defects, but show a reduced ability of neutrophils to migrate into the peritoneum after injection with TG. P-selectin-deficient mice have a pronounced defect in neutrophil accumulation at early times after TG injection (2 hr) but appear to have relatively normal rates of neutrophil migration into the peritoneum at later times, suggesting that an additional molecule(s) can substitute for P-selectin function at these times.

E-selectin also appears to play some role in leukocyte trafficking in a number of inflammatory processes. E-selectin gene transcription is normally not detectable in resting endothelial cells, but is strongly and rapidly induced by a variety of inflammatory mediators, including interleukin-1 (IL-1), tumor necrosis factor, interferon γ, substance P, and LPS (for reviews see Paulson, 1992; Bevilacqua, 1993). E-selectin expression is also associated in vivo with a number of disease states in humans and in experimentally induced disease in animals. In humans, expression has been observed in endothelium of inflamed skin in psoriasis, contact dermatitis, and delayed-type hypersensitivity reactions (Cotran et al., 1986; Picker et al., 1991), arthritic joints (Koch et al., 1991), and in heart and renal allografts undergoing rejection (Taylor et al., 1992; Allen et al., 1993; Brockmeyer et al., 1993). A variety of studies have shown that accumulation of neutrophils in disease models can be inhibited by monoclonal antibodies (MAbs) to E-selectin. Anti-E-selectin antibodies have been shown to inhibit neutrophil accumulation in models of peritonitis and lung inflammation in the rat (Mulligan et al., 1991) as well as in delayed-type hypersensitivity in the skin and late phase airway obstruction of nonhuman primates (Gundel et al., 1991; Silber et al., 1994). However, similar to the observations seen with the P-selectin-deficient mice, blocking of E-selectin function often only partially blocks the ability of neutrophils to enter sites of inflammation.

In addition to its role in inflammation, E-selectin function has been implicated in a number of other processes. Unactivated memory T cells can bind cells expressing E-selectin and have been suggested to be involved in the recruitment of this T cell subset (Shimizu et al., 1991). E-selectin expression has been observed in cutaneous vessels of inflamed skin and unactivated human CLA^+ T cells and bovine γδ T cells that home to the skin can bind E-selectin (Picker et al., 1991; Walcheck et al., 1993), suggesting that E-selectin may act as a tissue-specific homing receptor. Recently, antibody studies have also suggested a role for E-selectin in angiogenesis (Nguyen et al., 1993).

Mice deficient in E-selectin expression have been developed by targeted disruption of the E-selectin gene in embryonic stem (ES) cells to define the roles of E-selectin in leukocyte trafficking and development. The E-selectin mutation resulted in no obvious changes in neutrophil trafficking, suggesting that another molecule may substitute for E-selectin. Administration of an antibody to murine P-selectin effectively blocked neutrophil immigration in two distinct models of inflammation while having no effect in wild-type mice. These data demonstrate that neutrophil extravasation in these models is selectin dependent, and can be mediated by either P- or E-selectin, suggesting that the two selectins are functionally redundant.

Results

Generation of E-Selectin-Deficient Mice

To introduce a null mutation into the E-selectin locus, a replacement targeting vector was prepared as shown in Figure 1. The structure of the murine E-selectin gene has been described and is similar in organization to the E-selectin genes of other species (Becker-Andre et al., 1993). The LEC domain, encoded by a single exon, is absolutely required for E-selectin function and encodes the segment of the protein that interacts with the carbohydrate-containing ligand(s) (Erbe et al., 1992). The knockout construct contains an MC1–*neo* gene (Thomas and Capecchi, 1987) inserted after amino acid 162 within the LEC domain. This insertion disrupts the carbohydrate-binding LEC domain. The vector contains approximately 12 kb of 5′ and 3′ homology to the E-selectin locus, as well as a herpes simplex virus thymidine kinase (*tk*) gene for negative selection (Mansour et al., 1988). This vector was used to transform W9.5 ES cells (Szabo and Mann, 1994) and

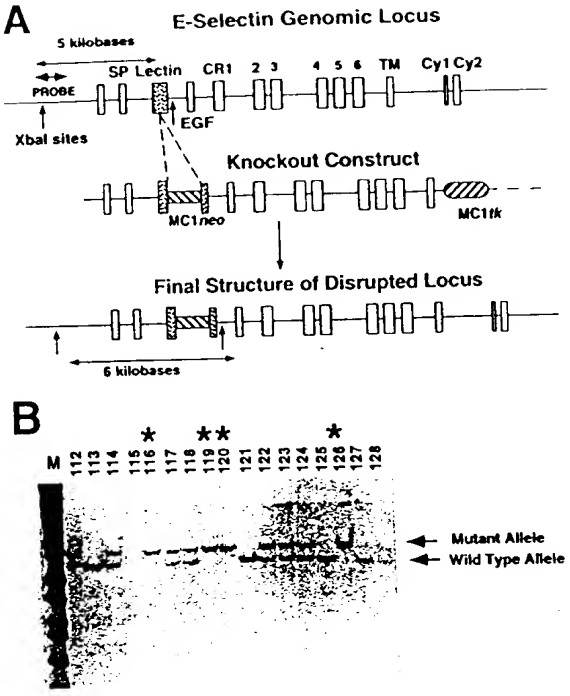

Figure 1. Disruption of the Murine E-Selectin Gene

(A) Structure of the murine E-selectin locus, knockout construct, and disrupted locus. Exons encoding the signal peptide (SP) lectin domain (LEC), EGF domain, CR elements, transmembrane (Tm), and cytoplasmic domains (Cy) are drawn as boxes. The LEC domain is indicated as a stippled box. The MC1–*neo* cassette and MC1–*tk* cassette are indicated by a hatched box and hatched oval, respectively. The locations of XbaI sites are shown by vertical arrows. The location of the probe used for genomic screening of ES cells is indicated by the double-headed arrow, as are the sizes of XbaI fragments predicted for the wild-type and disrupted alleles.

(B) Identification of mice homozygous for the disrupted E-selectin allele. Tail DNA from offspring of matings between two chimeric founder males derived from ES cell line 4.3.4 and their respective daughters were digested with XbaI and analyzed as described in the text. The location of bands derived from the wild-type and mutant alleles are indicated on the right. Progeny homozygous for the disrupted E-selectin gene are indicated by asterisks.

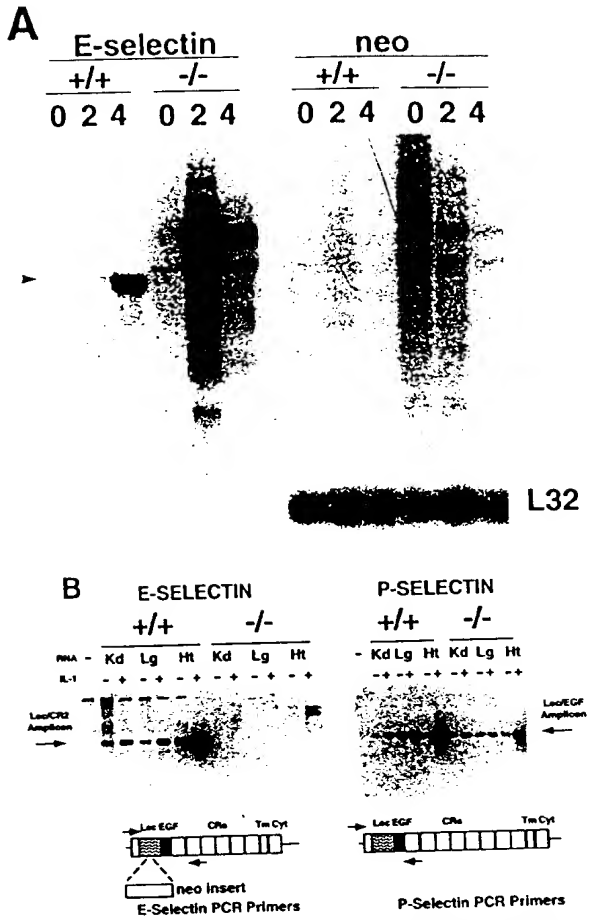

Figure 2. Analysis of E-Selectin mRNA by Wild-Type and E-Selectin Mutant Animals

(A) Wild-type (+/+) and E-selectin mutant (–/–) littermates were given LPS intranasally as described in the text. RNAs from lungs of mice harvested at 0, 2, or 4 hr after LPS administration were analyzed by Northern blot as described in the text. The Northern blot was sequentially hybridized to probes from the murine E-selectin gene, the *neo* resistance gene, or an L32 ribosomal protein gene as indicated. The location of the wild-type-sized E-selectin mRNA is indicated by an arrow on the left.

(B) RNA from kidney (Kd), lung (Lg), and heart (Ht) from mice injected with 1 μg of recombinant human IL-1α were analyzed by RT–PCR as described in the text. The location of the primers used for the analysis are shown on the bottom relative to their origin in the murine E-selectin and P-selectin cDNAs. Only a small region of the 3′ untranslated regions is shown for the cDNAs. The locations of amplicons predicted from the wild-type cDNAs are indicated by arrows. After electrophoresis and transfer to nylon membane, the PCR products were hybridized to specific LEC–EGF probes. Both autoradiographs represent 30 min exposures on Kodak XAR-5 film. The large band present in the samples form the wild-type mouse represents amplification of genomic E-selectin gene seqeunces from contaminating tissue DNA.

G418-resistant FIAU-insensitive colonies were isolated. As shown in Figure 1, the wild type and mutated E-selectin genes can be distinguished by Southern blot analysis of XbaI-digested ES cell DNA using a probe derived from sequences upstream of those present in the targeting vector. This probe detects a 5 kb fragment or a 6 kb fragment corresponding to the wild-type and mutant alleles, respectively. Approximately 70% of the isolated ES cell colonies contained the mutated E-selectin allele (data not shown). Two of these clones (4.3.4 and 4.5.1) were used to produce chimeric animals by injection into C57BL/6J blastocysts. Both clones gave rise to highly chimeric males that transmitted the mutant allele to their offspring.

Heterozygous animals from both lines were intercrossed to produce homozygous mice. Although comparable results were obtained with both lines, only results from animals generated with clone 4.3.4 are presented. Southern blot analysis, as shown in Figure 1B, was used to genotype offspring from the heterozygous animals. Mice homozygous for the mutant allele were detected at the expected Mendelian frequency. No gross abnormalities were detected in the organs of the heterozygous or homozygous animals. The homozygotes were of normal size and weight, and both sexes were fully fertile.

To determine that animals homozygous for the E-selectin insertion mutation were deficient in E-selectin expression, the production of E-selectin mRNA in either the homozygous animals or wild-type littermates were examined. Northern blot experiments were performed using RNA from the lungs of mice that were administered LPS intranasally to induce expression of the endothelial selectins (Figure 2A). Both the wild-type and homozygous E-selectin (−/−) animals produced E-selectin RNA in response to LPS. Although similar amounts of message were observed in both the wild-type and mutant mice, only the wild-type animals produced an RNA of the size reported for the murine E-selectin gene. The homozygotes produced a variety of alternatively sized messages but no wild-type-sized RNA. The structures of the different mRNAs produced by the mutant E-selectin gene are not known. However, the predominant RNAs were approximately the size predicted for an E-selectin RNA containing the *neo* gene insert. A *neo* probe also hybridized to most, if not all, the alternatively sized E-selectin RNAs found in the homozygous lungs, indicating that the RNAs produced in the homozygotes contained the *neo* insert. Both the wild-type and homozygous animals produced similar levels of P-selectin RNA upon induction with LPS (data not shown). Thus, the mutation alters the structure of E-selectin messages that are produced but does not effect the efficiency of transcription at the E-selectin locus or expression of the P-selectin gene.

Owing to the complex array of E-selectin RNAs produced in the homozygotes, it would be difficult to rule out the production of a small amount of functional E-selectin RNA by removal of the *neo* insert by rare undetected splicing events. To confirm that no mRNAs containing intact LEC and EGF coding regions were produced by the E-selectin (−/−) mice, RNAs were subjected to polymerase chain reaction (PCR) analysis after production of cDNA by reverse transcription (RT–PCR). In this experiment, expression of the endothelial selectins was examined in a variety of tissues 4 hr after injection of recombinant human IL-1α. As shown in Figure 2B, RT–PCR analysis detected similar levels of normal P-selectin message in both wild-type and homozygous mice. E-selectin amplicons of the size expected for wild-type E-selectin mRNA were readily detected from all tissues of the wild-type mouse. RT–PCR from the homozygotes carried out with E-selectin primers corresponding to the LEC and CR2 coding regions flanking the site of the *neo* insertion failed to detect any transcripts in which the *neo* insertion was removed. The only PCR products observed from homozygous mice were present in very low amounts and were approximately 1 kb larger than that predicted for the wild-type E-selectin RNA, apparently derived from an RNA containing the *neo* insert. The small amount of amplified DNA corresponding to the E-selectin–*neo* fusion transcript may reflect inefficient reverse transcription or PCR amplification of the longer RNA.

E-Selectin-Deficient Mice Produce No Detectable E-Selectin Protein

Hearts from LPS-treated mice were isolated and metabolically labeled with [^{35}S]methionine ex vivo. Proteins extracted from the labeled hearts were immunoprecipitated with antibodies to murine E-selectin, P-selectin, and VCAM-1. As shown in Figure 3, all three adhesion proteins were detected in wild-type mice but only P-selectin and VCAM-1 were detected in the E-selectin (−/−) siblings. Similar results were obtained when E-selectin was immunoprecipitated with three independent MAbs, demonstrating that the inability to detect E-selectin in this assay was not due to loss of a single epitope (data not shown). The amounts of P-selectin detected were similar in both knockout and wild-type animals, confirming that expression of P-selectin was not affected by the E-selectin mutation. Similar amounts of L-selectin were also detected by FACS analysis on the surface of the leukocytes of both wild-type and E-selectin-deficient animals (data not shown).

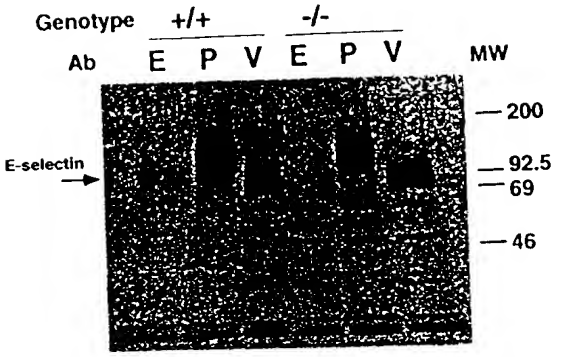

Figure 3. Analysis of Ex Vivo Labeled Adhesion Molecule Synthesis in Hearts of Wild-Type and E-Selectin (−/−) Mice

Proteins from ex vivo labeled heart tissue from mice injected with LPS were immunopreciptated with antibody to either murine E-selectin (E), P- selectin (P), or VCAM-1 (V) as described in the text. The location of wild-type-sized E-selectin protein is indicated by an arrow on the left and the location of size markers shown on the right.

Immunohistochemistry was also used to examine E-selectin expression in the hearts of LPS-treated wild-type and E-selectin (−/−) animals, as shown in Figure 4. E-selectin protein could not be detected in the endothelium surrounding the heart valve of a mouse homozygous for the E-selectin mutation, although E-selectin was readily detected in a similar section from a wild-type mouse. The presence of endothelial cells in the section was readily established by staining an adjacent section with an antibody to murine PECAM, which is expressed by all continuous endothelial cells (Muller et al., 1989; Albeida et al., 1990). Similar results were also observed in the lungs of the same animals (data not shown). These data suggest that no detectable E-selectin protein was produced in the E-selectin (−/−) mice, although the expression of VCAM-1 and P-selectin was relatively normal. In summary, E-selectin protein and functional E-selectin mRNA were not detected in animals homozygous for the mutant E-selectin allele, demonstrating that these animals were completely and selectively deficient for E-selectin expression.

Neutrophil Influx into the Peritoneum Is Dependent on the Activity of Either Endothelial Selectin

To determine whether E-selectin plays a role in neutrophil

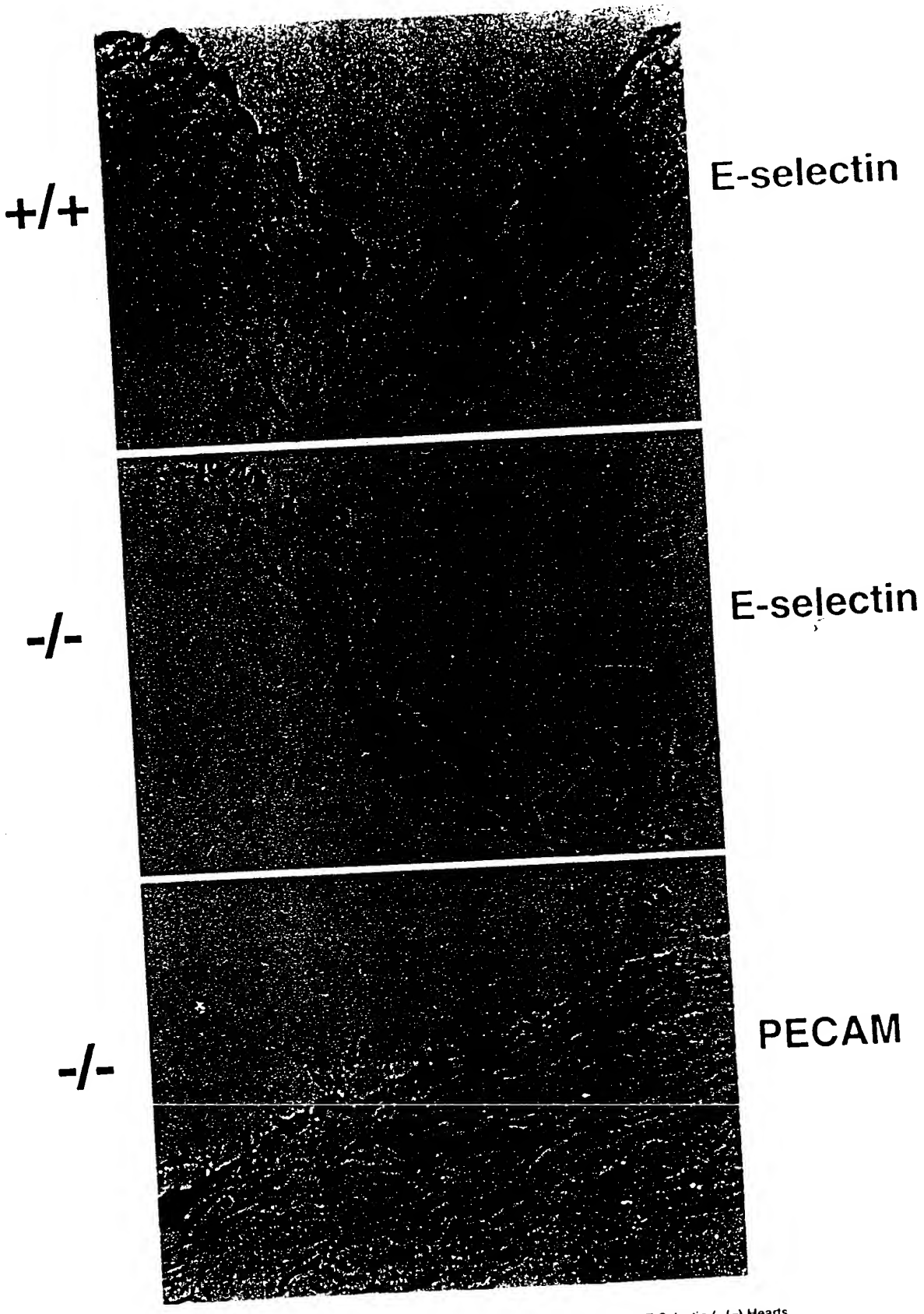

Figure 4. In Situ Immunohistochemical Analysis of E-Selectin Expression in Wild-Type and E-Selectin (−/−) Hearts

Hearts were isolated from LPS-injected mice and stained with antibody to murine E-selectin or murine PECAM as described in the text. The genotypes of the mice are indicated on the left and the antibody specificity on the right. Similar results were also obtained when expression was examined in the lungs of the same animals (data not shown).

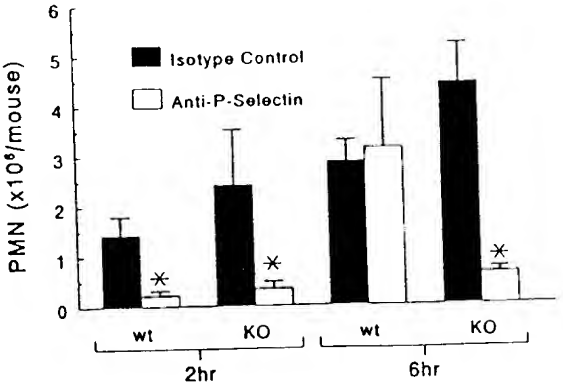

Figure 5. TG-Induced Peritonitis Requires Either P-Selectin or E-Selectin

The average numbers of PMNs found in the peritoneum of control E-selectin (+/+) (wild-type) and (−/−) mice (KO) are shown after 2 or 6 hr postinjection with TG. Mice were injected intravenously with saline containing an irrelevant isotype-matched control antibody (closed bars) or the anti-murine P-selectin antibody, 5 H1 (open bars). The bars represent data (mean and SEM) from groups of four mice. Statistically significant differences ($P < 0.05$) are indicated with asterisks.

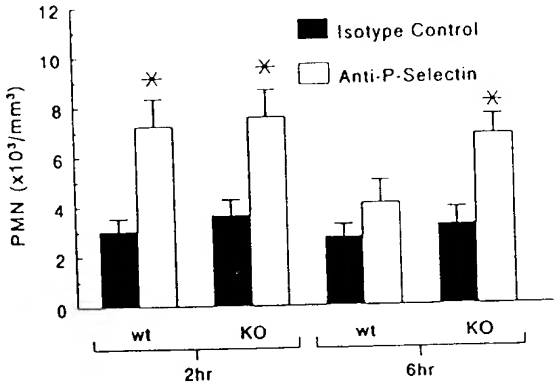

Figure 6. Blockade of Neutrophil Trafficking Results in an Increase in Circulating Neutrophils

The number of circulating neutrophils from the animals used in Figure 5 are shown. The data shown are the mean values with SEM. Animals were either treated with a control irrelevant antibody (closed bars) or the anti-P-selectin MAb, 5H1 (open bars). Statistically significant differences ($P < 0.05$) are indicated by asterisks.

immigration mediated by inflammatory stimuli, the number of neutrophils entering the peritoneum was measured at various times after injection with TG. The E-selectin deficiency by itself had little effect on TG-induced peritonitis. No differences in the numbers of peritoneal neutrophils present in wild-type or E-selectin-deficient mice were observed at early times (2 hr) or late times (6 hr) after TG injection. These data suggest that in the mouse, either E-selectin plays only a small role in neutrophil trafficking, or the lack of E-selectin can be compensated for by the expression of another molecule. Since endothelial cells express two highly related selectin molecules (E- and P-selectin) in response to inflammatory mediators, the influx of neutrophils in the E-selectin-deficient mice may be due to the presence of functional P-selectin protein. In this regard, it is important to note that neutrophil influx into the peritoneum induced by TG after blockade of either P-selectin or L-selectin function is impaired primarily at early times after induction with TG. The partial blockade of neutrophil trafficking by inhibition of P-selectin or L-selectin function may be explained in several ways. One explanation is that the endothelial selectins are functionally redundant. Alternatively, neutrophil influx might be independent of selectin function at later times.

To test the hypothesis that either P-selectin or E-selectin can mediate neutrophil influx at later times in this model, the response to TG was determined in wild-type and E-selectin (−/−) mice after the inhibition of P-selectin function using a MAb, 5H1, specific for murine P-selectin. The results shown in Figure 5 demonstrate that neutrophil influx at 2 hr after TG induction was P-selectin dependent in both groups of mice. Treatment with 5H1 reduced the number of neutrophils in the peritoneum by approximately 85% in wild-type and E-selectin-deficient mice. The effect of 5H1 antibody was very similar to the effect of disruption of the P-selectin gene. The number of neutrophils entering the peritoneum was reduced by 6.5-fold in P-selectin-deficient mice (Mayadas et al., 1993) and by 7-fold by the anti-P-selectin MAb. These results confirm the observation made in P-selectin-deficient mice that early immigration of neutrophils is largely dependent on P-selectin. The observation that the 5H1 antibody had an equal effect on both groups of mice suggested that there was little or no contribution by E-selectin at the 2 hr timepoint.

The results obtained at 6 hr after the induction of peritonitis with TG indicated that either E-selectin or P-selectin can mediate neutrophil immigration. In contrast with experiments carried out at 2 hr after TG injection, 5H1 had no effect on the influx of neutrophils in wild-type mice at the later timepoint. The same antibody treatment, however, reduced the number of peritoneal neutrophils in the E-selectin-deficient mice by 85%. The reduction in neutrophil counts cannot be due to nonspecific effects of the 5H1 antibody, since the antibody only affects immigration in the E-selectin (−/−) mice. These data demonstrate that P-selectin can compensate for the loss of E-selectin in the knockout mice at later times in TG-induced peritonitis and that E-selectin can compensate for the loss of P-selectin in the wild-type mice treated with 5H1. Furthermore, these results have recently been confirmed in studies with wild-type mice treated with a combination of anti-E-selectin and anti-P-selectin MAbs (K. W. M. et al., unpublished data).

As the effects of 5H1 treatment could also be explained by a reduction in the number of peripheral neutrophils, the number of circulating neutrophils present in the blood of the test animals was also determined. Blockade of neutrophil accumulation in the peritoneum, however, was accompanied by a significant increase in the number of peripheral neutrophils (Figure 6). At the 2 hr timepoint, when P-selectin blockade was sufficient to inhibit neutrophil accumulation, 5H1 treatment resulted in a 2-fold increase in the concentration of peripheral neutrophils in both groups of mice. 5H1 treatment of wild-type mice did not

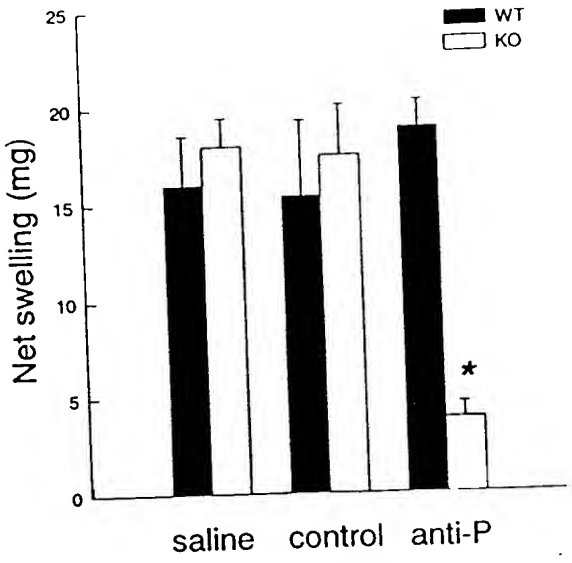

Figure 7. Delayed-Type Contact Hypersensitivity Response Requires the Function of Either E-Selectin or P-Selectin

The data shown represent the relative swelling in response to oxazolone challenge observed in the ears of sensitized wild-type or E-selectin (−/−) as measured by the net increase in the weight of biopsies as described in the text. Animals were given an intravenous injection of saline alone or saline containing a control MAb or the anti-P selectin antibody 5H1 as indicated in the figure. Treatment of the E-selectin (−/−) animals with 5H1 resulted in an approximately 80% reduction in swelling. Each group consisted of 4–8 age- and sex-matched animals. The results shown are the mean values ± SEM.

significantly affect circulating levels of neutrophils at the 6 hr timepoint. Blockade of P-selectin function with 5H1 in the E-selectin-deficient mice, however, resulted in a 2-fold increase in peripheral blood neutrophils. These data are consistent with a requirement for one of the endothelial selectins in the regulation of neutrophil trafficking in this model of acute inflammation.

Function of Either P-Selectin or E-Selectin Is Required for a Contact Hypersensitivity Response in the Skin

The results from the peritonitis model suggested that the majority of neutrophil migration were dependent on the function of one or the other of the two endothelial selectins. To determine whether the dependence on selectin function and the redundancy of the two selectins were specific for the peritonitis model or was a more general phenomenon, the roles of E-selectin and P-selectin were examined in a model of delayed-type contact hypersensitivity in the skin. Mice were sensitized to oxazolone and subsequently challenged by application of oxazolone to the ear. Figure 7 illustrates the resulting swelling observed in wild-type and knockout mice as measured by the increase in the weight of standard size skin biopsies. The ear swelling in response to oxazolone was not statistically different in the wild-type and E-selectin (−/−) mice. Treatment of the wild-type mice with the anti-P-selectin antibody 5H1 had no effect on swelling, while treatment with the same antibody reduced swelling by 80% in the E-selectin-deficient animals. Histological analyses of frozen sections of the ear biopsies were examined to determine whether the reduction in ear swelling was accompanied by a similar reduction in the infiltration of leukocytes. As shown in Figure 8, the ears of the wild-type and E-selectin-deficient animals were similarly edematous and had large numbers of infiltrating leukocytes after challenge with oxazalone. Large accumulations of neutrophils were also observed in pustules (Figure 8, shown by arrows) at similar frequencies in both groups of animals. No significant differences in the histology of the ears of wild-type mice treated with either 5H1 or an irrelevant control antibody were observed. Treatment of the E-selectin (−/−) animals with 5H1, however, dramatically reduced the swelling of the skin and the number of infiltrating leukocytes observed in the sections. The formation of neutrophil-containing pustules was also completely eliminated by by 5H1 treatment of the E-selectin-deficient mice. In fact, the ears of the (−/−) mice treated with 5H1 were almost identical in appearance to those animals that had been challenged with oxazolone without prior sensitization, suggesting that the swelling observed was due to the nonspecific irritant effects of the oxazalone (compare Figures 8A and 8E). In addition, antibody treatment of these animals did not result in a nonspecific reduction in circulating neutrophils (data not shown). These data again demonstrated that infiltration of neutrophils requires the function of an endothelial selectin, which can be supplied by either E-selectin or P-selectin. These data have also been confirmed using a combination of antibody to murine E-selectin and P-selectin in wild-type mice (P. C. W. et al., unpublished data). These results demonstrate that the edema and cellular infiltration observed in this model of skin inflammation was dependent on the endothelial selectins.

Discussion

This report describes the characterization of E-selectin-deficient mice. Mice lacking a functional E-selectin gene were viable, fertile, and of normal size, indicating that the E-selectin gene was not required for normal development. Although a recent study reported that antibody to bovine E-selectin prevented capillary formation by endothelial cells in culture, no defects in vascularization were observed in the E-selectin-deficient animals (data not shown). Potential effects of the E-selectin mutation on neovascularization in adult mice are currently being examined. The lack of developmental alterations in the E-selectin-deficient mice is consistent with the observation that E-selectin is only expressed in endothelial cells upon activation by inflammatory mediators. However, as demonstrated in this paper, a role for the selectins in development cannot yet be ruled out, owing to an overlapping of function of the endothelial selectins for leukocyte trafficking. Demonstration of a role for the selectins in vascularization or in other aspects of development may require the simultaneous inactivation of the P-selectin and E-selectin genes.

Numerous previous studies have suggested a role for the selectins in neutrophil trafficking. These studies uti-

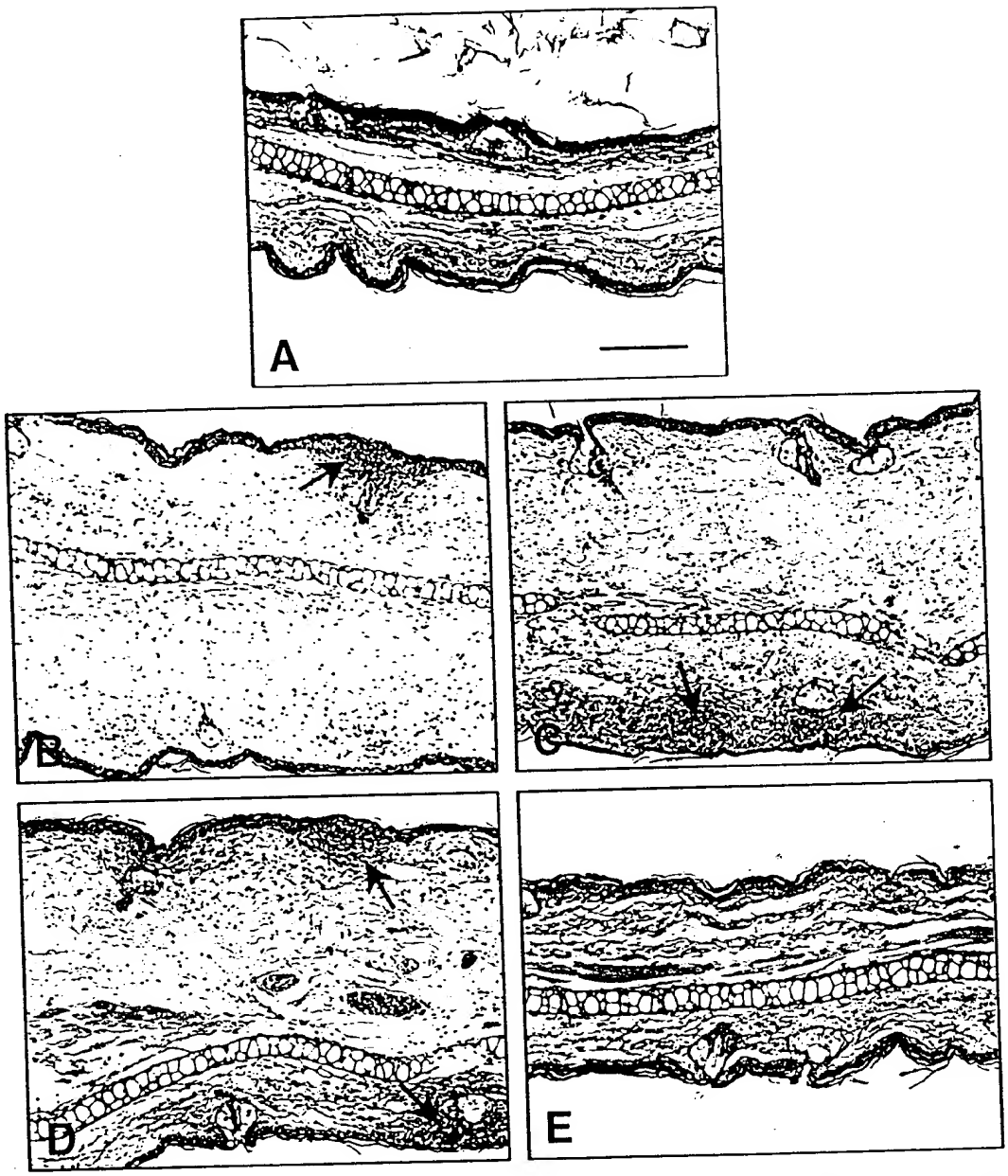

Figure 8. Histological Examination of the Delayed-Type Hypersensivity Response in Wild-Type and E-Selectin-Deficient Animals

Representative sections from the ear biopsies obtained in the delayed-type hypersensivity experiment presented in Figure 7 are shown. (A) represents an ear section from a wild type mouse that received the challenge of oxazolone to the ear without prior sensitization and represents the small inflammatory response from the nonspecific irritant effects of oxazolone. (B) and (D) represent sections from wild-type or E-selectin (−/−) mice treated with 8G9, respectively. (C) and (E) represent sections from wild-type or E-selectin (−/−) animals, respectively, treated with the anti-P-selectin antibody 5H1. The size bar in (A) represents 144 μM. The accumulations of neutrophil in pustules are indicated by arrows.

lized MAbs, immunoglobulin chimeric proteins, or targeted gene disruption to block the activity of single selectin gene products. Such studies, however, often reported only partial inhibition of neutrophil migration or inflammation. The data presented in this paper demonstrate that E-selectin can act as a major mediator of inflammation. E-selectin was able to mediate the trafficking of neutrophils at late times in TG-induced peritonitis. This is shown clearly by the inability of the anti-P-selectin antibody, 5H1, to block neutrophil influx in wild-type mice 6 hr after TG injection, while the same antibody treatment effectively blocked influx in the E-selectin-deficient mice. This result cannot be due to an incomplete block of P-selectin function by 5H1, since the antibody treatment appeared to block the accumulation of neutrophils 2 hr after injection of TG as effectively as disruption of the P-selectin gene in P-selectin knockout mice (Mayadas et al., 1993). Similar results have recently been obtained in nontransgenic animals using antibody to both murine E-selectin and P-selectin (unpublished data). The results presented in this paper also dem-

onstrate that P-selectin can mediate neutrophil influx at later times in the model of peritonitis, as demonstrated by the ability of 5H1 to block neutrophil influx in the absence of E-selectin in the knockout mice. Thus, these results demonstrate that either P-selectin or E-selectin can mediate the influx of neutrophils and suggest that the two molecules are redundant or highly overlapping in function.

Our data are consistent with the results from previous studies that suggest that neutrophil influx at early times in TG-induced peritonitis is P-selectin dependent. The dependence on P-selectin function at 2 hr after TG injection is likely, because P-selectin is the predominant selectin present on the surface of endothelial cells at that time. P-selectin is stored in Weibel–Palade bodies and is rapidly transported to the surface of activated endothelial cells, while E-selectin is synthesized de novo. Because murine P-selectin is also transcriptionally activated by cytokines and LPS, endothelial cells are likely to express high levels of both selectins at late times after induction with TG. Although our data demonstrate that both E-selectin and P-selectin can mediate neutrophil migration in the same model inflammatory responses, it is not known whether, or how often, the two molecules are coexpressed in other species. It should be noted that blockade of E-selectin function alone, using anti-E-selectin antibody, has been reported to efficiently block some model inflammatory responses in other species (Mulligan et al., 1991). A likely interpretation of these observations, in consideration of the data presented here, is that E-selectin expression is induced in the absence of P-selectin in such settings.

An overlapping of function of the endothelial selectins was also demonstrated in a model of skin inflammation. The data obtained using oxazolone-induced delayed-type hypersensitivity were comparable to those observed at late times in TG-induced peritonitis. Neither swelling nor neutrophil accumulation was affected by either the E-selectin mutation in the knockout mice or by the P-selectin antibody in wild-type mice. However, both swelling and cellular accumulation were greatly reduced by 5H1 treatment of the E-selectin (−/−) animals. These data again demonstrate that the inflammatory process can be mediated by either endothelial selectin. The cellular infiltrates observed in the ears after the delayed-type hypersensitivity response contained monocytes and lymphocytes as well as neutrophils. The dramatic reduction in the numbers of all infiltrating cells after blocking both selectins suggests that the selectins may be required for trafficking of a wide variety of cell types, although the effect of selectin blockade on some of these cell types may be indirect. The effect of selectin blockade on trafficking of different leukocyte populations during both acute and chronic inflammatory reactions is currently under investigation.

Several alternative explanations might also explain the data presented in this paper. One alternative explanation to the observation that 5H1 blocks neutrophil accumulation in the E-selectin-deficient animals might be antibody-induced neutropenia. This seems unlikely, since 5H1 had no effect in wild-type mice in the delayed-type hypersensitivity model or at later times in the model of peritonitis. It is possible, although unlikely, that this antibody had a specific neutropenic effect in the E-selectin (−/−) mice. However, measurement of circulating neutrophils in all mice examined in these models demonstrated that administration of 5H1 caused a large increase in the number of circulating neutrophils (Figure 6; data not shown). Thus, the reduction of cellular infiltration in the (−/−) mice caused by 5H1 was not due to a reduction in the number of circulating neutrophils. A final explanation might be that the results are peculiar to the strain of mice used in these experiments or are peculiar to the ES cell–derived mice. This is unlikely, as the results of recent experiments indicate that neutrophil accumulation can be efficiently blocked during peritonitis and delayed-type hypersensitivity using a combination of blocking MAbs against murine E-selectin and P-selectin in nontransgenic mice of other strains (unpublished data). Thus, the overlapping function of the two endothelial selectins appears to be a general phenomena at least for all the mouse strains currently examined and is not dependent on ES cell–derived animals.

In conclusion, we have demonstrated that the majority of neutrophil influx in two distinct models of acute inflammation required the expression of endothelial selectins. In either model, the majority of neutrophil accumulation was inhibited by blocking the function of the endothelial selectins. These observations are consistent with multistep models of leukocyte trafficking in which the initial interaction with and rolling of leukocytes on the endothelium are mediated by selectins. It is important to note that a small amount of neutrophil accumulation occurred (15% of wild type) even when the function of both endothelial selectins was blocked. It is possible that this low level of neutrophil migration is independent of selectins. Alternatively, the residual neutrophil migration may be mediated by L-selectin expressed on the surface of mouse neutrophils. These two mechanisms are now being tested by utilizing blocking L-selectin MAbs in the E-selectin-deficient mice.

The ability of both endothelial selectins to mediate neutrophil accumulation in two distinct models of inflammation suggests that the overlapping function of E-selectin and P-selectin is not peculiar to a single model of inflammation but is a general phenomenon. If this is true, then the degree of leukocyte accumulation in a given disease setting may depend on the overall concentration of selectin molecules on endothelial cells, rather than expression of a single selectin. This notion is particularly relevant to the recent efforts to develop selectin–antagonist–based anti-inflammatory drugs. The results presented in this paper suggests that the most clinically effective selectin antagonists may be those that are active against multiple selectins.

Experimental Procedures

Construction of the Targeting Vector

Murine E-selectin genomic clones were isolated from λ libraries derived from either 129SV DNA (Stratagene, Incorporated) or BALB/c DNA (Clonetec, Incorporated). Inserts were subcloned and analyzed using standard procedures. A 1.8 kb SacI fragment from pBS/SV153 encompassing the LEC domain was subcloned into pBSΔSma-2 yielding pBSΔSma–SacI. An XhoI–SalI *neo* resistance cassette from pMC1–*neo*pA (Stratagene, Incorporated) was then cloned into AvaI-digested pBSΔSma–SacI flushing of all ends with Klenow fragment

of DNA polymerase I (New England Biolabs, Incorporated), creating pBS/KO-A. The SacI fragment from pBS/KO-A was then subcloned into the KpnI site of pNEB/NotI, creating pNEB/LecNeo-9. The insert from pNEB/LecNeo-9 was removed with AscI and XbaI and inserted into similarly digested pNEB/NotI, creating pKbAsc-1. A 5.3 kb XbaSalI fragment of E-selectin genomic sequence was then inserted into XbaI–SalI-digested pKbAsc-1, creating pmk3–1. A 1 kb HindIII–SacI fragment of 5′ E-selectin genomic sequences from pM7H5 (derived from BALB/c DNA) was inserted into the AscI site of pmk3–1 after blunting of the fragment overhangs creating pBSV-2. Finally, a *tk* cassette was created by insertion of a BstBI–SalI fragment from p14322 (HSV–*tk*) containing the *tk* coding region into BstBI–SalI-digested pMC1–*neo*pA*, creating pMC1–*tk*. An XhoI–SalI *tk* cassette from pMC1–*tk* was then inserted into the PmeI site of pBSV-2 after filling in the fragment overhangs, creating the final targeting vector, pBSVTK-10.

Identification of Targeted ES Cell Clones and Production of Mice

The ES cell line, W9.5 was propagated on primary embryonic feeder cells as previously described (Abbondanzo et al., 1993) in media containing leukemia inhibitory factor and 15% fetal calf serum (GIBCO BRL, Incorporated). Feeder cells were mitotically inactivated by irradiation. Of the ES cells from an 80% confluent 225 cm^2 flask, one third were transformed by electroporation with 25 μg of pBSVTK-10 that had been linearized with NotI. Electroporation and selection of G418- and FIAU-resistant ES cell colonies was as previously described (Stewart et al., 1992). Individual colonies were isolated 7–10 days after electroporation. ES cell cultures in 48-well dishes were grown to confluency and then split. Of the ES cells, two thirds were used to produce DNA for analysis and the remaining cells frozen for later use. ES cell and tail DNAs were prepared as previously described (Laird et al., 1991).

DNA was digested with XbaI, fractionated by agarose gel electrophoresis, and transferred to nylon membranes using a Hoeffer electroblot apparatus. Membranes were hybridized with a PCR-generated E-selectin probe. The probe consists of unique E-selectin sequences (data not shown) just upstream from the most 5′ sequences present in the targeting vector. E-selectin probe was produced using a Prime-it kit as described by the manufacturer (Stratagene, Incorporated).

ES cell clones containing a targeted E-selectin allele were then karyotyped. Two clones, 4.3.4 and 4.5.1, containing a 40 XY karyotype, were used to produce chimeric mice by injection of the clones into C57BL/6J blastocysts and implanted into the uterine horns of pseudopregnant C57BL/6 × CBA/J F1 females. Chimeric males were bred to C57BL/6J females. Progeny containing the mutant E-selectin allele were intercrossed. Both lines yielded homozygous E-selectin (−/−) pups at an approximately 25% frequency. The results presented are from mice of mixed 129sV and C57BL/6J backgrounds. Only data for the 4.3.4-derived mice are shown, but similar results were also obtained for the 4.5.1 line. For many experiments, homozygous wild-type and E-selectin-deficient siblings produced from heterozygous intercrosses were bred individually to generate the large numbers of animals needed for these experiments.

RNA Analysis

RNAs were prepared from frozen tissues using RNAZOL B as described by the manufacturer (Biotex Laboratories, Incorporated). Of total RNA, 10 μg was used to produce first-strand cDNA using Superscript RT according to the instructions of the manufacturer (GIBCO BRL, Incorporated). A portion of the cDNA was then used for PCR analysis. PCR primers for murine E-selectin were directed to the LEC domain (TAGATTCTGAAGCTCCAACTCGCCT) and to the CR2 domain (GTTTGCCTTCCCCTCTGACTC), resulting in a 700 bp amplicon for the wild-type E-selectin mRNA. PCR primers for murine P-selectin amplify a 580 bp segment using primers from the signal sequence (GACTCCAGGATCCCAAAAGGTT-CCTGGACGCCAAGACTC) and a primer derived from the EGF domain (TGAGATGGTCGACGCGTATT-CACACTCTG-GCCCATAGAAGCC). PCR reactions were carried out using Taq polymerase according to the instructions of the manufacturer (Boehringer-Mannheim Biochemical, Incorporated). PCR products were fractionated on a 1% agarose gel, transferred to a nylon membrane, and hybridized to either murine E-selectin or P-selectin probes as described above. All filters were washed for several hours at 65°C in 0.2× SSC before exposure to Kodak XAR-5 film.

Antibody Production

Rat MAb to murine E-selectin (9A9.E3) was recently described (Norton et al., 1992). A similar strategy was utilized to generate MAbs to murine P-selectin and the details will be described elsewhere (C. R. N. et al., unpublished data). In brief, sequences encoding the murine P-selectin LEC and EGF domains were amplified by PCR and fused to sequences encoding the first two CR domains of human E-selectin. Recombinant fusion protein (MuHu P-selectin) was affinity purified from transfected COS cell supernatants using an anti-human E-selectin (anti-CR1–2) MAb 9A1 sepharose column. The isolated protein was used for immunization of rats and production of MAbs. Anti-murine P-selectin antibodies 5H1 and 10A10 recognize native P-selectin induced by tumor necrosis factor on murine eEnd.2 endothelial cells and 5H1 blocks the adhesion of HL60 cells and bone marrow–derived neutrophils to recombinant murine P-selectin. MAb 5H1 does not crossreact to either murine E-selectin or L-selectin.

Immunoprecipitation of Ex Vivo Labeled Protein

Five wild-type and five E-selectin (−/−) mice were injected intraperitoneally with phosphate-buffered saline (PBS) containing 100 μg of LPS (Sigma, Incorporated). Mice were sacrificed and hearts removed 3 hr after LPS injection, pooled, minced, and placed in 2 mL of labeling media (cysteine-free DMEM [GIBCO BRL, Incorporated], 10% dialyzed fetal calf serum) containing 2.5 mCi [^{35}S]cysteine (Amersham, Incorporated). Tissues were then incubated for 3 hr at 37°C, pelleted, and washed once with PBS. Extracts were made by homogenizing the tissue in CHAPS lysis buffer (50 mM Tris–HCl [pH 8.0], 0.3 M NaCl, 10 mM CHAPS, 1 mM PMSF, 10 mM iodoacetamide). Lysates were cleared by centrifugation at 10,000 × g for 30 min at 4°C. Supernatants were divided equally into three samples and immunoprecipitated with either MAb 10A10.C1 (rat anti-murine P-selectin), 9A9.E3 (rat anti-murine E-selectin), or MK/2.7 (rat anti-murine VCAM-1; Miyake et al., 1991). Samples were incubated at 4°C for 1–2 hr. Goat anti-rat IgG agarose beads were used to precipitate any complexed proteins. Bound proteins were eluted from the agarose beads by incubation for 15 min in 50 μl of 0.1 M glycine (pH 2.8), 0.15 M NaCl. Finally, samples were analyzed by gel electrophoresis (Laemmli, 1970) using a 5%–15% gradient gel. Gels were treated with Amplify (Amersham, Incorporated) prior to autoradiography.

Immunohistochemistry

Four hours after an intraperitoneally injection of 1 mL of sterile saline containing 100 μg LPS (from Escherichia coli serotype 055:B5, Sigma Chemical Company, St. Louis, Missouri), three knockout and three wild-type mice (7- to 8-week-old males) were sacrificed via a lethal injection (0.2 mL, intraperitoneally) of Euthanasia 5 Solution (Henry Schein, Incorporated). They were then exsanguinated by aortic transection. Sections of the heart were embedded in OCT in cryomolds and snap frozen. Cryostat sections (6–7 mm) were prepared, air dried onto silane-coated slides (Cel-Tek, Incorporated, Glenview, Illinois), fixed in cold (4°C) acetone for 5 min, and air dried again. All subsequent steps were carried out at room temperature. Cryosections were rehydrated in PBS for 15 min prior to a 30 min incubation in 1% H_2O_2 in methanol to block endogenous peroxidase. The slides were loaded onto an automated immunostainer (Cadenza, Shandon Scientific, Limited, Runcorn, United Kingdom) for the remainder of the immunostaining procedure. The sections were allowed to react with 10% normal rabbit serum in PBS for 30 min followed by 15 min each in avidin and biotin blocking solutions (Vector Laboratories). Anti-murine E-selectin antibody, 9A9, or an anti-murine PECAM antibody (provided by S. Albelda) was used for primary staining. An irrelevant isotype-matched rat MAb was used as a negative control at the same concentration (data not shown). Sections were incubated with MAb 9A9 or the irrelevant control for 2 hr followed by a 30 min incubation with biotinylated rabbit anti-rat IgG (Vector Laboratories) using 50 μL of the secondary antibody diluted in 10 mL of PBS containing 150 μL of normal rabbit serum. The slides were then incubated for 30 min in an avidin–biotin–peroxidase conjugate as suggested by the manufacturer (ABC Elite Kit, Vector Laboratories). Antibody binding was visualized with 3,3′-diaminobenzidine tetrahydrochloride (ISOPAC, Sigma Chemical Com-

pany), mixed 1:2 with 35 μL H_2O_2 in 50 mL of distilled water, and incubated for 5 min. The slides were then rinsed in water and counterstained with hematoxylin.

Peripheral White Blood Cell Counts

Mice were bled from the retro-orbital sinus into EDTA-anticoagulated tubes while under CO_2 sedation. White blood cell counts were determined using a Coulter cell counter (Coulter Electronics, Incorporated) following the lysis of red blood cells. Blood smears were prepared and stained with Wright's stain for differential leukocyte counts.

TG-Induced Peritonitis Assay

Male mice of each geneotype were injected intraperitoneally with 0.5–1 ml of 3% TG. Mice were sacrificed by cervical dislocation after sedation with CO_2 either 2 or 6 hr after injection. Peritoneal exudates were collected by lavage with 4 mL of Hank's balanced salt solution containing 10% fetal calf serum. Total cell counts were determined with a Coulter cell counter. Differential leukocyte counts were performed on Wright's-stained cytocentrifuge smears. A minimum of 200 cells per sample were counted. Total neutrophil counts were determined from the total number of exudate cells multiplied by the percentage of neutrophils.

Delayed-Type Contact Hypersensitivity Assay

Sex-matched animals were used at 7–9 weeks of age. Each group represents the results from 4–8 mice. Procedures were based on those reported by Chapman et al. (1986). For sensitization, abdomens were anesthetized with lidocaine followed by the application of 1.5 mg of oxazolone in 10 μl of 97% acetone and 3% DMSO. Animals were injected intravenously with 100 μL of sterile saline or sterile saline containing either 200 μg of the 5H1 anti-P-selectin MAb or 200 μg of an irrelevant isotype-matched control antibody, 8G9 5–7 days later. After each intravenous injection, animals were challenged by treatment of ears with lidocaine followed by application of 5 μL of vehicle containing 100 μg of oxazolone in acetone–DMSO to each side of the treated ears. As a control, the other ear received the same dose of vehicle without oxazolone. At 24 hr after the challenge, animals were anesthetized with Metofane (Pitman-Moore, Mundelein, Illinois) and sacrificed by cervical dislocation. Skin punches (8 mm) were obtained from vehicle and oxazolone-treated ears and weighed to the nearest 0.1 mg. The results reported as net swelling are differences between the vehicle alone and the vehicle- and oxazolone-treated ears. Ear biopsies were fixed in 10% buffered formalin, sectioned, and stained with hemotoxylin and eosin for histology or were snap frozen in O. C. T. for use in immunohistochemistry.

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